THE DIGESTIBILITY OF CAROTENE BY RATS AND CHICKENS

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ONE FIGURE

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It has been known for some years that carotene has vitamin A potency when it is fed to animals. It is also known that carotene or cryptoxanthin are responsible for all the vitamin A potency of plants or plant products. Now that carotene can be determined chemically in a comparatively short time, work can be done on its properties and relations which was difficult when only biological methods were available. Since carotene must be digested before it can be utilized for vitamin A potency, a knowledge of its digestibility and the factors which affect it, is of considerable importance. Little work has been done on the digestibility of carotene. Whitnah, Peterson, Atkesen and Cave ('37) have reported that a large amount of carotene was excreted in the feces of cows and that from 79 to 210% of the carotene consumed was recovered in the feces. The authors made no explanation of the excessively high amounts of carotene recovered from the feces, but it is probable that the supposed carotene determined in the feces was contaminated with other yellow pigments. Wilson, Das Gupta and Ahmad ('37) reported that carotene eaten by humans in raw or cooked carrots or cooken spinach was about 90% absorbed when the diet contained fat and only 50% absorbed when the fat was omitted. Van Eikelen and Pannevis ('38) also report that fat aided the absorption of carotene.

EXPERIMENTAL

Rats or chickens which had been receiving a diet containing liberal amounts of carotene were first placed upon a diet containing negligible amounts of carotene (approximately 0.1 part per million). The excrement was collected daily and the amount of carotene determined by a method already described by Fraps and Kemmerer ('37). The quantity of coloring matter measured as carotene in the excrement decreased until after 2 to 3 days when it became constant and remained constant until the work was discontinued or the diet changed. It is evident from the procedure followed that most of this

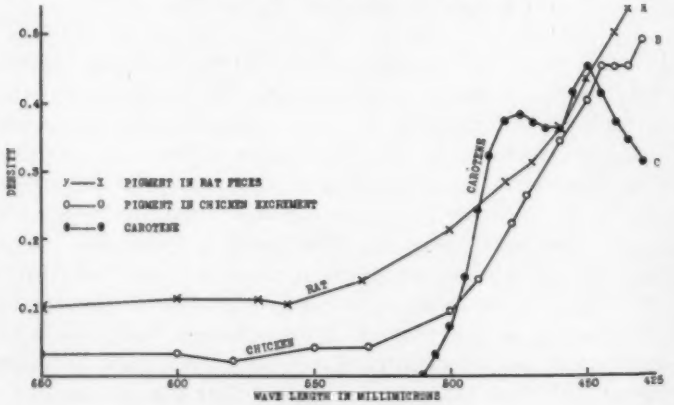

Figure 1

coloring matter was not carotene but metabolic products of the animal. This theory is confirmed by the absorption curves. The absorption curves at a level equivalent to 0.5 parts of carotene per million of petroleum ether were obtained with the use of a Bausch and Lomb spectrophotometer and are compared with the absorption curve for carotene in figure 1. The absorption curve for the yellow coloring matter from the excrement of rats is different from the curve of that found in the excrement of chickens, and both curves are different from the curve for carotene. It is clearly evident that very little if any of this yellow color is due to carotene.

The digestion experiments were planned so that correction could be made for the non-carotene coloring matter. Six adult male rats or seven half grown White Leghorn chickens (two males and five females) were placed in wire cages with wire bottoms arranged so that excrement and uneaten feed could be quantitatively collected. These animals were first fed the basal ration until analysis showed that the quantity of yellow color in the excrement (calculated to carotene) was constant. A known quantity of carotene in alfalfa leaf meal or dissolved in oil was next added to the basal ration and this mixture fed for 3 days. Then finally the basal ration was fed until the yellow color content of the excrement again became constant. The rat excrement then contained from 1.2 to 1.4 parts per million of color expressed as carotene, and the chicken excrement from 0.4 to 0.5 parts per million. These quantities were subtracted from the results of the analyses, and the remainder was considered to be derived from the carotene. This procedure was justified for the reason that all the conditions were constant except the small amounts of the carotene carrier which was fed during the digestion period. The method of procedure and details of an experiment on a group of six adult male rats can be seen in table 1.

It was found that the excrement, collected daily, kept well in cold storage so that after the preliminary work the excrement from 2 days collection was combined for the estimation of carotene. The basal ration used for rats was composed of white corn meal 74%; wheat gray shorts 10%; casein 10%; irradiated yeast 4%; salt 1% and bone meal 1%. For chickens it contained white corn meal 59%; wheat gray shorts 25%; casein 12%; calcium carbonate 1%; tricalcium phosphate 1%; irradiated yeast 1% and salt 1%.

RESULTS OF THE DIGESTION EXPERIMENTS

The amount of carotene which disappeared in the passage of the feed through the digestive tract of the animal was considered to be the amount of carotene digested. Some of the carotene apparently digested may have been destroyed during the process of digestion.

TABLE 1

Procedure and details of an experiment with a group of six adult male rats

	DAY RATION WAS FED	AMOUNT RATION EATEN	AMOUNT FECES COLLECTED	CAROTENE IN FECES PARTS PER MILLION	CAROTENE IN FECES LESS CORRECTION (1.3 PARTS PER MILLION)	CAROTENE TOTALS
		gm.	*gm.*			*micro-grams*
Preliminary feeding	1	50.0				
period in which ration	2	50.0	7.54	12.7		
containing no carotene	3	50.0	9.16	5.7		
was fed	4	60.0	5.67	1.3		
	5	60.0	8.99	1.3		
	6	60.0	7.38	1.1		
	7	60.0	7.48	1.3		
	8		6.60	1.4		
Period in which ration	1	59.2				
containing 19.9 parts	2	59.0	10.04			
per million carotene	3	59.1	13.22			
was fed	Total	177.3	23.26	57.0	55.7	1295.6
Period in which ration	4	60.0	18.46			
containing no carotene	5	60.0	6.26			
was fed	Total		24.72	63.5	62.2	1537.6
	6	60.0	11.31	7.3	6.0	67.9
	7	60.0	5.24	2.6	1.3	6.8
	8		6.21	1.3	0.0	
Total carotene excreted						2908.0
Total carotene fed (177.3 × 19.9)						3528.0
Carotene digested						620.0
Per cent carotene digested						17.6

Such quantities of carotene were fed as would approximate those contained in rations used in practical feeding. The percentages of carotene digested from dehydrated alfalfa leaf meal at several different levels of intake is given for twelve experiments with rats and eight with chickens in table 2. The tests were duplicated with all of the five levels fed rats and with three of the levels fed chickens, and the agreements between the duplicate results are satisfactory. When the amount of carotene in the diet was 20 parts per million of the

feed, the digestibility of carotene by rats was 18 to 23% and when the carotene in the diet was decreased to 1.2 parts per million, the digestibility increased to 43%.

The growing chickens digested carotene to a greater extent than did the adult rats. When 20 parts per million of carotene were fed, the rats digested 18 to 23% and the chickens 29%. When 1 part per million was fed the rats digested 43% and the chickens 69%.

TABLE 2

Digestibility of carotene by rats and chickens

	CAROTENE IN FEED PARTS PER MILLION	CAROTENE DIGESTED
		%
Adult male rats fed dehydrated alfalfa leaf meal	19.9	17.6
	20.0	22.9
	10.5	22.4
	10.0	28.1
	6.2	33.9
	6.3	31.5
	2.6	31.4
	2.1	30.4
	1.2	43.1
	1.2	43.2
Adult male rats fed carotene in Wesson oil	16.1	48.3
	10.5	51.2
Growing chickens fed dehydrated alfalfa leaf meal	20.3	29.1
	10.8	29.4
	10.2	25.1
	5.2	37.0
	2.3	53.9
	2.2	49.1
	1.3	68.9
	1.1	63.9

The data in table 2 show that carotene is much more highly digested when it is dissolved in Wesson oil and added to a feed than when it is fed in alfalfa leaf meal. Rats digested 22.4% carotene from 10.5 parts per million in the feed from alfalfa leaf meal, and 51.2% from a similar quantity of carotene dissolved in Wesson oil. This difference in digestibility

is understandable when we remember that the carotene fed in the alfalfa leaf meal must be brought into solution, and some of it may be protected by plant material, while the carotene in oil is already dissolved in a medium which is highly digestible. According to Fraps ('28) fat in cottonseed meal may be digested by chickens to the extent of 86.2%, in corn 85.1%, and in soy bean oil meal 81.4%. These figures would indicate that carotene may be digested to a less extent than the oil in which it is dissolved.

According to Sherwood and Fraps ('35) hens receiving three to four biological units (2 to 3 micrograms of carotene) per gram of feed stored 25% of the vitamin A potency in their eggs. If the 25% stored in the eggs is compared with 37 to 49% digestibility by growing chickens, we find the possibility of a high utilization of the digested carotene.

The apparent digestibility of carotene and therefore the utilization of carotene for vitamin A potency depends on the quantity of carotene fed, the nature of the material in which it is contained, the kind of animal, and other factors. Therefore, for experimental work on carotene in which feeding tests are involved, this digestibility must be considered.

SUMMARY

1. Yellow pigments were found in the excrement of rats and chickens which had received feed containing negligible amounts of carotene. These coloring materials could not be separated from the carotene by the usual chemical procedures. The absorption curves of these pigments were quite different from that of carotene, even though they absorbed some light in the same region of the spectrum as carotene. Corrections were made for these pigments in estimating the digestibility of carotene.

2. The percentage of carotene apparently digested depended on the quantity of carotene fed, on the nature of the material in which it was contained and on the kind of animal to which it was fed. When carotene in dehydrated alfalfa leaf meal was fed at the rate of 20 parts per million, the rats digested

18 to 23% and the chickens 29%. When 1 part per million was fed the rats digested 43% and the chickens 69%. When carotene in Wesson oil was fed at the rate of 10.5 parts per million of the feed, the rats digested 51% as compared with 22% when an equal amount of carotene was fed in dehydrated alfalfa leaf meal.

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THE PRODUCTION OF MANGANESE RICKETS IN RATS

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During the course of investigations on the effects of various metallic elements on bone formation, a group of young rats was fed a ration containing manganese chloride. Roentgenographic examination of these animals revealed what appeared to be rachitic changes in the bones. Attention was thus directed to the possibility that manganese compounds might possess a rachitogenic action, and experiments were carried out to determine this point.

EXPERIMENTAL

The rats used in these studies were healthy growing animals, reared on a stock ration (Bills, Honeywell, Wirick and Nussmeier, '31). They were placed on the respective diets when they were 4 weeks old and weighed between 42 and 55 gm. In table 1 are given the composition and calcium and phosphorus contents of the principal or type diets.

[1] Deceased.

[2] Aided by a grant from Mead Johnson and Company, Evansville, Indiana.

TABLE 1

Composition of principal diets

A. Stock diet (optimal Ca and P)		B. Rachitogenic diet (high Ca-low P)	
Yellow corn	76.0	Yellow corn	76.0
Crude casein	5.0	Gluten	20.0
Linseed oil meal	16.0	NaCl	1.0
Alfalfa meal	2.0	$CaCO_3$	3.0
NaCl	0.5		
$CaCO_3$	0.5	Ca, 1.240%; P, 0.250%; Ca: P, 5: 1.	

3 parts diet + 1 part whole milk powder.
Ca, 0.475%; P, 0.450%; Ca: P, 1.1: 1.

C. Control diet (low Ca-low P; only 0.5% $CaCO_3$, approximately)

Yellow corn	76.0
Gluten	20.0
NaCl	1.0
$CaCO_3$	0.5

Ca, 0.246%; P, 0.256%; Ca: P, 1: 1.

Preliminary experiment. In the preliminary experiment the effect of manganese on bone formation was investigated by adding manganese dioxide (MnO_2) and manganese chloride ($MnCl_2 \cdot 4\,H_2O$) to the stock diet in amounts which gave the rations a manganese content of 1%. By the twenty-sixth day on the diets the animals were growing slowly or had ceased to grow, and one member of the manganese chloride group had died. In view of the harmful effect of the diets, the experiment was terminated and roentgenograms were made of the tibiae of the rats. The data and results are shown in table 2.

It is seen from the table that the animals receiving the soluble manganese chloride developed moderate to severe rickets, whereas the group receiving manganese dioxide, insoluble in water and not readily soluble in gastric juice, showed only slight rarefaction of the bones. The production of rickets by addition of a soluble manganous salt to a stock ration suggested a rachitogenic action of the Mn^{++} ion, although the possible effect of the additional chloride was not excluded.

Production of rickets with manganese carbonate. Following the indication that manganese chloride could produce

rachitic changes in bones, the rachitogenic potency of manganese was investigated by substituting 2.9% of manganese carbonate (which is equimolar with 2.5% calcium carbonate) for 2.5% of the calcium carbonate in a high calcium-low phosphorus rachitogenic diet (compare table 1, diet B). The control diet in this case was the low calcium-low phosphorus modification of the rachitogenic diet (compare table 1, diet C). This modified ration, with only 0.5% approximately of calcium carbonate added instead of the usual 3.0%, is not rachitogenic, although productive of a slight or moderate degree of osteo-

TABLE 2

Preliminary experiment with manganese compounds

GROUP	DIET	NO. OF ANIMALS	DAYS ON DIET	WEIGHT CHANGE (AV.)	Ca OF DIET	P OF DIET	Ca : P RATIO	ADDED Mn	ROENTGENOGRAM
				gm.	%	%		%	
1	Stock	2	26	+89.0	0.475	0.450	1.1 : 1	0.0	Normal
2	Stock 98.4 MnO_2 1.6	5	26	+19.2	0.467	0.443	1.1 : 1	1.0	Slight rarefaction
3	Stock 96.4 $MnCl_2 . 4\ H_2O$ 3.6	5	26	+13.4	0.458	0.434	1.1 : 1	1.0	Moderate to severe rickets

porosis (Shelling, Asher and Jackson, '33). At the same time the original experiment with stock diet was repeated, but 2.9% of manganese carbonate, equal to the amount used with the low calcium-low phosphorus ration, was added instead of manganese chloride.

The rats were maintained on the diets for 21 days, after which roentgenograms of the tibiae were made, and representative specimens of the bones were taken for histological study. In table 3 are given the details of these experiments.

The results of these experiments demonstrated that the substitution of 2.9% of manganese carbonate for 2.5% of the calcium carbonate in a standard rachitogenic diet produces

what may be conveniently referred to as 'manganese rickets,' approximately equal in severity to the rickets developed on the unsubstituted calcium carbonate ration. Furthermore, the addition of manganese carbonate to a stock diet with an optimal calcium and phosphorus content also produced rickets, although the phosphorus in the diet was much higher than in the rachitogenic ration.

TABLE 3

Experiments with manganese carbonate

GROUP	DIET	NO. OF ANIMALS	DAYS ON DIET	WEIGHT CHANGE (AV.)	Ca OF DIET	P OF DIET	Ca : P RATIO	ADDED Mn	ROENTGENOGRAM	HISTOLOGY
				gm.	%	%		%		
4	Control (Low Ca-low P)	7	21	+24.0	0.246	0.256	1 : 1	0.0	Normal, or slight osteoporosis	Slight to moderate osteoporosis
5	Control 97.5 $MnCO_3$ 2.9	18[1]	21	— 4.0	0.239	0.249	1 : 1	1.4	Moderate to severe rickets	Severe rickets (4 animals)
6	Stock	3	21	+75.7	0.475	0.450	1.1 : 1	0.0	Normal	Normal
7	Stock 97.1 $MnCO_3$ 2.9	14	21	+21.7	0.461	0.437	1.1 : 1	1.4	Moderate to severe rickets	Severe rickets (4 animals)

[1] One animal died after 15 days on the diet.

Chemical analyses of pooled sera from group 4 and from group 5 yielded the following results:

Group 4
(control, low Ca-low P) Ca = 7.1 mg. per cent, P = 8.0 mg. per cent, Ca × P = 56.8

Group 5
(control + $MnCO_3$) Ca = 9.2 mg. per cent, P = 3.3 mg. per cent, Ca × P = 30.4

These values show that with the low phosphorus diet the manganese carbonate caused a reduction in serum inorganic phosphorus, similar to that produced by calcium carbonate in the high calcium-low phosphorus type of rickets.

Spectrographic examinations of whole blood were made to determine the approximate manganese concentration. The blood manganese was found to be slightly increased in the manganese-fed animals, but it did not exceed the order of magnitude of 0.1 mg. per cent, an insignificant amount in comparison with the serum calcium and phosphorus values. The tibiae of the manganese-fed animals also showed a small increase over the trace of manganese present in the tibiae of the stock rats, but the extent of manganese deposition in the bone seemed slight in comparison with the amount fed.

Effect of vitamin D upon 'manganese rickets.' The efficiency of vitamin D in curing 'manganese rickets' was determined by supplementing the diet of rachitic rats with 2% of

TABLE 4

Effect of vitamin D on 'manganese rickets'

GROUP	DIET		NO. OF ANIMALS	DAYS ON DIET	WEIGHT CHANGE AVERAGE	ROENTGENOGRAM (AFTER VITAMIN D)	HISTOLOGY
					mg.		
5-D	Control	97.5	8	10	−8.5	Slight	Slight
	$MnCO_3$	2.9				healing	healing
	Viosterol	200 I.U.					(4 animals)
7-D	Stock	97.1	5	10	−0.2	Good	Good
	$MnCO_3$	2.9				healing	healing
	Viosterol	200 I.U.					(5 animals)

Viosterol 1 D (diluted to this strength with olive oil), equivalent to 200 I.U. of vitamin D per 100 gm. of ration. The animals were fed the rachitogenic diet for 21 days and the Viosterol supplement for an additional 10 days. Before the administration of the vitamin the presence of rickets was demonstrated roentgenographically, and at the end of the supplementary period the degree of healing was ascertained both roentgenographically and histologically. The data regarding these groups of animals are given in table 4.

The appreciable loss of weight in the group of rats receiving the low calcium-low phosphorus ration (group 5-D) suggests a possible toxicity which must be remembered in the interpretation of the results. Despite the loss in weight, which in

itself might be conducive to healing, the animals showed only slight healing of their rickets with an amount of vitamin D in excess of the usual curative dose. This amount of vitamin D, when fed to rachitic animals along with the standard calcium carbonate rachitogenic diet, is sufficient to induce nearly complete healing in a 10-day period. The slight degree of the healing in the animals fed the manganese carbonate-substituted ration presented some evidence, therefore, of a decreased efficiency of vitamin D in curing 'manganese rickets,' but the effect of diminished food consumption must also be considered. However, the experiment with the stock diet (group 7-D) indicated that vitamin D promoted effective healing of 'manganese rickets' when the phosphorus content of the diet was not low.

DISCUSSION

Shipley, Park, McCollum and Simmonds ('22) described the production of rickets by diets either high in calcium and low in phosphorus, or low in calcium and high in phosphorus. Further investigation has shown that experimental rickets may also be produced by incorporating into the diet any one of the following elements: strontium (Stoeltzner, '08; Shipley, Park, McCollum, Simmonds and Kinney, '22); magnesium (McCollum, Simmonds, Shipley and Park, '23); thallium (Buschke and Peiser, '24); iron (Waltner, '27); beryllium (Branion, Guyatt and Kay, '31); lead (Shelling, '32); and aluminum (Deobald and Elvehjem, '35). Lecoq and Villette ('34) studied the inhibiting action of certain metals upon the curative effect of adding di-sodium phosphate to the diet of rats previously made rachitic. It was reported that the calcification ordinarily observed upon the addition of di-sodium phosphate was manifestly retarded by the simultaneous administration of the carbonates of bismuth and calcium, but even more so by the carbonates of iron, magnesium, strontium and manganese.

The experiments which have been presented in this paper demonstrate that manganese, in manganous compounds which

are easily soluble in the gastro-intestinal tract, is capable of producing rickets. The amounts of manganese used, however, were far greater than those which would be found in foodstuffs. The manganous ion, like the other rachitogenic cations previously mentioned, forms an insoluble phosphate. Presumably the essential factor in the production of rickets by manganese is the formation of the relatively insoluble and poorly absorbable manganese phosphate in the intestinal tract, with a consequent decrease in the amount of phosphorus available for bone structure. Indeed, Becker and McCollum ('38) have recently observed practically normal growth and good reproduction in rats fed a diet containing about 1% of manganese (3.6% of $MnCl_2 \cdot 4\ H_2O$) but also containing vitamin D and a relatively high amount of phosphorus. It thus appears that availability of phosphate decreases the deleterious effect of manganese.

SUMMARY

1. 'Manganese rickets' has been produced in rats by the substitution of an equimolar amount of manganese carbonate (2.9%) for 2.5% of calcium carbonate in a high calcium-low phosphorus rachitogenic diet, and also by the addition of 2.9% of manganese carbonate to a stock diet of optimal calcium and phosphorus content.

2. A soluble manganese salt, manganous chloride, was rachitogenic, but the more difficultly soluble manganese dioxide was not.

3. Vitamin D was effective in curing 'manganese rickets' when added to a diet optimal in calcium and phosphorus, but only slightly effective when added to a diet low in both calcium and phosphorus.

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SPECTRUM ANALYSIS FOR TRACE ELEMENTS IN THE ASHES OF HUMAN, GOAT AND COW MILK

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Twenty-five years ago Osborne and Mendel ('13) directed attention to the requirements of animal cells not only for energy but also for suitable constructive materials. They stated that such substances as enzymes, internal secretions, etc., may require either special antecedent products, chemical activators or minute amounts of easily overlooked rarer elements or compounds.

Five years later Osborne and Mendel ('18) reported some effects of inorganic elements in nutrition.

During later years much attention has been called to the importance of very minute amounts of certain inorganic elements as a result of numerous investigations upon food requirements of plants, animals and lower forms of life.

For recent reviews on the importance of trace elements in animal nutrition, reference is made to Hart and Elvehjem ('36), and in plant nutrition to Mozé ('36).

As milk is one of the important foods, human and goat milk were investigated for trace elements in addition to cow milk already reported by Drea ('34). The importance of including goat milk is due to the fact that some individuals are allergic to cow milk and not to goat milk or vice versa. The three kinds of milk were secured from individuals drinking from the same municipal source of drinking water.

Waters from different geographical areas vary in their contents of trace elements and this is of importance to the physiology and structure of plants and animals. Harmful as well as beneficial effects may result. As an example of a harmful effect may be cited the presence of fluorine in drinking water to the amount of 2 p.p.m. The enamel defect known as 'mottled' enamel is produced when children drink a sufficient amount of this water. It has also been shown by Boissevain and Drea ('33) that teeth and bones of human beings drinking this water contain more fluorine than do similar tissues from individuals living on the Atlantic coast of this country. The substitution of a drinking water containing less than 0.5 p.p.m. fluorine has resulted in 'mottled' enamel not developing in children of a region where 'mottled' enamel was previously prevalent with the former water supply, which contained 6 p.p.m. fluorine (McKay, '33).

MATERIAL

The milks from six nursing women and four goats were investigated. The ten samples were examined separately. Each sample from the previously cleaned nipples was directly placed in a thoroughly cleaned Pyrex flask after the first small amounts had been thrown away.

METHOD OF INVESTIGATION

Spectrum analyses of the ashes by means of direct current excitation between graphite electrodes were made according to the method previously described (Drea, '34). Three or four spectra were produced for each ashed sample. Quantitative determinations were not made. Only the inorganic elements that can be determined by this method are reported. For example, the halogens, with the exception of fluorine, cannot be demonstrated. Fluorine, when calcium is present, can be detected because of the molecular CaF_2 band at $\lambda 5291$ provided at least 0.01% fluorine is present.

RESULTS

The findings plus those for the cow milk and drinking water are recorded in table 1.

Aluminum, barium, boron, copper, iron, lead, silver, strontium, titanium and vanadium were present in very small amounts in all samples of the human, goat and cow milk. Chromium was present in five of six human samples, seven of twelve cow samples and in all four goat samples. Fluorine was not demonstrated in any milk though it was present

TABLE 1

TRACE ELEMENTTS	HUMAN MILK 6 SAMPLES	GOAT MILK 4 SAMPLES	COW MILK 12 SAMPLES	DRINKING WATER
Al	+	+	+	+
Ba	+	+	+	+
B	+	+	+	+
Cr	5 of 6+	+	7 of 12+	+
Cu	+	+	+	+
F	—	—	—	+
Fe	+	+	+	+
Pb	+	+	+	+
Li	5 of 6+	+	+	+
Mn	3 of 6+	—	—	+
Mo	+	—	+	+
Rb	+?	+	+	—
Si	+?	+?	+?	+
Ag	+	+	+	+
Sr	+	+	+	+
Sn	2 of 6+	—	3 of 12+	—
Ti	+	+	+	+
V	+	+	+	+
Zn	5 of 6+	+	+	+

Ca, Mg, P, K and Na were present in the milks in the usual larger amounts. With the exception of P they were also in the water.

in the drinking water to the extent of 2 p.p.m. Lithium was present in five of six human samples and in all of the others. Manganese was present in three of six samples of human milk and was absent from the others. Molybdenum was present in the human and cow milks and was absent from the goat milk. Rubidium was questionably present in the human milk and was present in the other milks. Silicon was questionably present in all of the milks. Tin was demonstrated

in two of the six samples of human milk, in three of twelve cow milk samples and not in the goat milk. Zinc was present in five of six samples of human milk and was present in all other milks. Any of the elements that can be detected by this spectrographic method and not reported as present were either absent or present in too small amounts to be detected.

Comparison with another basic food, the yolks and whites of hen eggs investigated by Drea ('35) shows that aluminum, barium, copper, iron, strontium, titanium and vanadium were present in all. The following elements were also present except as follows: boron was not detected in five egg whites; chromium in two of five egg yolks, three of five whites, one of six human milks and five of twelve cow milks; lead in one of five yolks and three of five whites; lithium in one of six human milks (not investigated in eggs); manganese in three human milks, all of goat and cow milks, and egg whites; molybdenum in goat milks and four of five egg yolks; rubidium in four of five egg whites and questionably in the human milks; silicon questionably in the milks; silver in five of five egg yolks and two of five whites; tin in four of six human milks, nine of twelve cow milks, all of the goat milks and eggs; and zinc in one of six human milks and five of five whites.

DISCUSSION

Attention is directed especially to chromium, fluorine, lead, manganese, molybdenum and silver.

Chromium was found in most of the milk samples. Zbinden ('31) reported it present but Wright and Papish ('29) and Blumberg and Rask ('33) found it was not evident.

Fluorine was present in the drinking water to the extent of 2 p.p.m. At no time has the fluorine of milk, blood, soft tissues of many organs of animal bodies including human beings drinking this water for more than 40 years, the tissues of plants, eggs (whites, yolks and shells) been demonstrated by means of the spectrograph. Two explanations are possible for this: 1) that much of the fluorine was volatilized during the ashing, and 2) too little fluorine was present to excite

the CaF_2 band when the substances were reduced to ashes even if volatilization of the fluorine had not occurred. That the second hypothesis is the correct one appears to be probable from the work of Philips, Hart and Bohstedt ('34). They determined that the range of fluorine in cow milk from cows on a control basal diet with its calculated naturally present daily 0.3064 gm. of fluorine per cow was from 0.07 to 0.22 mg. per liter with an average of 0.13 mg. The addition of rock phosphate to the basal diet resulting in the calculated addition of 1.6649, 2.5380 and 3.0825 gm. of fluorine daily per cow resulted in averages of 0.172, 0.126 and 0.192 mg. respectively of fluorine per liter of milk. Even the largest amount of fluorine noted in the milk, 0.26 mg. per liter, is too small to produce the enamel defect. These amounts of fluorine in the milk are also too small to demonstrate with the spectrograph method used as it is sensitive only to 0.02% fluorine in the ash. Boissevain and Drea ('33) have pointed out that ingested fluorine largely leaves the body in the urine and feces. Some, as pointed out before, is stored in the teeth and bones. However there is also this fact to be noted: considerable fluorine is stored in calcium deposits located in pathological organs, as has been demonstrated by the writer in spectrographic analyses of the ashes of diseased thyroid glands from human beings.

Lead was evident in all samples and Zbinden also reported it present. It was not reported by Wright and Papish or by Blumberg and Rask. Kehoe, Thamann and Cholak ('33) reported cow milk contained not to exceed 0.04 mg. lead per liter and that human milk contained comparable amounts. They consider these amounts of lead to be normal.

Manganese is of general physiological importance (von Oettengen, '35). Its presence in egg yolks and absence from egg whites is interesting. The chick is insured this element. Cow and goat milks contained too little of this element to be demonstrated and only three of six human milks were shown to contain it. Zbinden and also Wright and Papish reported it present but Blumberg and Rask considered evidence of

its presence to be questionable. The newborn child may not have sufficient manganese stored in its tissues, and it might be advisable to supplement a milk diet with a little manganese, especially where there was a prolonged lactation. Older individuals whose diet for some reason is largely milk may also profit by having manganese added to the diet. Wilgus, Norris and Heuser ('37) have directed attention to manganese in perosis.

Molybdenum was found in all samples of cow and human milk, though not in the goat milk. This element was not reported as present by Zbinden, Wright and Papish or Blumberg and Rask. It is widely distributed in tissues according to H. ter Meulen ('32). He stated that blood, bile, milk, eggs and sundry tissues contain 0.00014 to 0.00003 gm. per kilogram as determined by a colorimetric method.

Silver is reported as present in the human and goat milks as it was previously in the cow milk by Drea. Because this was not done by Wright and Papish and also not by Blumberg and Rask for cow milk it is suggested that the strong trace of silver in the drinking water was the source of this element in the milks here reported. The alfalfa eaten by the cows also contained a strong trace of silver.

SUMMARY

Spectrum analyses of human and goat milks for 'trace' elements have been made and compared with similar investigations of cow milk and hen eggs.

The importance of the drinking water as a part source for these trace elements is emphasized.

Special attention is directed to chromium, fluorine, lead, manganese, molybdenum and silver.

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THE EXCRETION OF VITAMIN B_1 IN THE URINE AND FECES

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It has been recognized for many years that vitamin B_1 is excreted in the urine and feces. Only recently have quantitative studies of the relation between the B_1 intake and the excretion of this substance been made. Leong ('37) and Harris and Leong ('36) studied the urinary excretion of vitamin B_1 in white rats, and the latter also studied humans. The bradycardia method of B_1 determination was employed. Knott ('36) made B_1 balance studies on children, the food and excreta being assayed by a rat growth method. Helmer ('35), Roscoe ('36) and others have determined the B_1 in feces and urine semiquantitatively. With the development of the simple and rapid method for B_1 determination by Schultz, Atkin and Frey ('37 a, b) it was felt that the problem could be efficiently investigated. While this work was in progress the results of Leong's investigation were published. Since new and interesting data had already been obtained, the work was continued.

PROCEDURE

The basal ration used for the rats was tested and found to be negative for vitamin B_1 by both the fermentation and rat growth methods. This is important since, as Schultz et al. ('37 b) have shown, 2-methyl-5-ethoxymethyl-6-amino pyrimidine (a substance used in the vitamin synthesis) also stimulates gas production under the conditions of their test. Feeding tests with this pyrimidine indicate that it is immediately and completely excreted in the urine.

Metabolism cages for the white rats consisted of simple cylindrical wire cages fitted on large glass funnels. An inverted round bottom flask set over a small beaker was used to collect the urine. Having the flask of larger diameter than the beaker caused the feces to be deflected, thus effecting a separation. Spillage of food to any great extent was prevented by carefully avoiding overfeeding. The urines were washed from the funnels, flasks and collection beakers each day, acidified, and made to volume. They were then sterilized in flowing steam for 30 minutes. The feces, after thorough maceration with water, were treated in the same manner. Aliquots of the solutions so obtained were used for vitamin assay.

PRELIMINARY EXPERIMENTS

A group of four neuritic rats was used for preliminary studies. The Bc-free [1] basal diet was composed of 62% rice starch, 14% hydrogenated cottonseed oil,[2] 18% vitamin-free casein, 2% cod liver oil, and 4% salts. The non-B_1 factors of the B complex were supplied in part by mixing 15 parts of a rice bran-rice polish residue with 85 parts of the Bc-free ration. The bran-polish residue was made by extracting the B_1 from the mixture at pH 4 in the cold. The more commonly used autoclaved yeast was not used in this work since experience had shown that the destruction of B_1 by alkaline autoclaving produced something equivalent to the above-mentioned pyrimidine fraction. A separate supplement of 20 γ riboflavin per day was given to each rat. The vitamin B_1 supplement, when given, was administered in the form of a 20% solution of the crystalline vitamin (Merck's synthetic).

Starting with a supplement of 25 γ B_1 per day, the output in the urine was found to average 3 γ per rat per day. After

[1] In this manuscript, the symbol 'Bc' is used to designate vitamin B complex, under which term we include all of the factors in yeast, i.e., B_1, B_2, riboflavin, nicotinic acid, as well as the filtrate factors, etc. The symbol 'Gc' is used to designate the vitamin G complex, under which we include all of these factors, known and unknown, except vitamin B_1.

[2] Crisco.

10 days no increase in urinary B_1 was observed and the supplement was increased to 50 γ per day. For 4 days at this level no significant increase in B_1 output was found, but by the sixth day the amount was quadrupled. From this point onward successive increases to levels of 100 γ and 200 γ produced a prompt response in the B_1 found in the urine. On the day following the introduction of the 100 γ level, for instance, the average B_1 content of the urine was 29 γ, and continuation of this level for 6 days caused no material change. In these preliminary experiments rather high daily variations were encountered and in further work 3-day composite samples were used wherever possible.

VITAMIN EXCRETION OF RATS RECEIVING SUPRA-MINIMAL B_1 SUPPLEMENTS

Two sets of four rats were used in this experiment, one of strain A and one of strain D, low and high B_1 requiring strains respectively (Light and Cracas, '38). In order to avoid possible complications due to an insufficiency of factors other than B_1 each rat was fed 0.5 gm. dry yeast per day to supply the Gc factors. The riboflavin supplement was discontinued and the Bc-free diet (without rice-bran polish) was fed ad libitum. Analysis showed that the 0.5 gm. of yeast contained the equivalent of 15 γ of B_1. The animals weighed 70 to 120 gm. at the start.

It was the object of this experiment to determine the excretion of the vitamin in both urine and feces only after the body of the animal had adjusted itself to a fixed intake level. The steady state reached at a given level is termed excretion equilibrium at that level. The animals were prepared for this series as follows: the nearly depleted rats were fed at the 15 γ level (0.5 gm. dry yeast) for 2 weeks and the vitamin content of the urine and feces measured periodically. When it had been determined that the figures given for weekly period 1 (see table 1) represented excretion equilibrium (15 γ) the B_1 supplement was increased. Thereafter samples were taken only on the last 3 days of each weekly period. Only single

week periods were used for the levels of 65 γ and above since the results obtained in the preliminary work had shown that at these levels the attainment of excretion equilibrium is very prompt.

The results given in table 1, under strains A and D, represent analyses of four 3-day composite samples averaged together to give one value for each strain per weekly period.

These growing rats, which may be considered normal, excreted a total of 10 γ of B_1 with an intake of 15 γ. Increasing

TABLE 1

The excretion of vitamin B_1

WEEKLY PERIOD	TOTAL B_1 INTAKE	AVERAGE B_1 EXCRETED PER RAT PER DAY							
		Strain A				Strain D			
		Urine	Feces	Total	Per cent	Urine	Feces	Total	Per cent
	γ	γ	γ	γ	γ	γ	γ	γ	γ
1	15 oral	5.4	5.1	10.5	70	4.75	5.5	10.2	70
2	40 oral	7.0	12.0	19	48	7.7	4.9	12.6	32
3	40 oral	9.4	14	23.4	59	9.0	5.4	14.3	36
4	65 oral	12.7	14.5	27.2	41	16.0	7.9	23.9	37
5	115 oral	37.5	30.5	68	59	34.5	21.4	55.9	49
6	215 oral	71	54	125	58	81	40	121	56
7	515 oral	165	115	280	54	157	110	267	53
8	500 injected	412	17.5	429.5	86	337	11.5	348.5	70
9	500 injected	375	17.5	392.5	78.5	435	13.5	448.5	90

intake levels of from 40 to 515 γ caused a proportionate increase in vitamin excretion. At these higher levels the excreta appear to contain about one-half of the daily intake. There is a tendency for urinary B_1 to be higher than fecal B_1, and this is particularly pronounced with strain D. This is the only difference between strains observed here.

The response to injection of the vitamin is radically different from the feeding results as shown by the excretion of about 80% of a 500 γ daily injection. Virtually all of this excretion is in the urine.

VITAMIN B_1 EXCRETION FOLLOWING 'SATURATION'

At the end of the above-described experiment the tissues of the rats receiving 515 γ B_1 per day were considered to be 'saturated' with the vitamin at this level. Since it is known that the body becomes depleted of vitamin B_1 rather rapidly, compared with other vitamins, it was thought worth while to follow the B_1 excretion when the supplement was dropped back to 0.5 gm. dry yeast (15 γ B_1). It should be noted that this constitutes depletion while still receiving adequate amounts of the vitamin. Table 2 gives the data obtained on four of the rats described in table 1, a pair for each strain.

TABLE 2

Influence of body stores on excretion of B_1

DAYS	TOTAL B_1 INTAKE	VITAMIN B_1 EXCRETED PER DAY					
		Strain A			Strain D		
		Urine	Feces	Total	Urine	Feces	Total
	γ	γ	γ	γ	γ	γ	γ
0	515	165	115	280	157	110	267
1– 2	15	30	21	51	30	13	43
3– 4	15	16	5.7	21.7	10	5.5	15.5
5– 6	15	11.7	5	16.7	8.7	5.0	13.7
7– 8	15	10	....		8.2	....	
9–10	15	8.3	8.7	17	7.6	7	14.6
11–12	15	8.2	....		8.6	....	
13–14	15	6.0	....		5.8	....	
15–16	15	6.2	6.2	12.4	5.0	5	10
17–18	15	7.5	....		5.8	....	
19–20	15	4.7	5.0		4.3	5	

The excretion of the vitamin dropped from 275 γ per day to 45 to 50 γ in the first 2 days. Within 2 weeks the rats appeared to be again in excretion equilibrium at the 15 γ level. Strain A rats seemed to retain the vitamin slightly better than strain D, and this may have significance with regard to the observed difference in B_1 requirements.

THE URINARY EXCRETION OF VITAMIN B_1 BY DOGS

While it is not convenient to use dogs in the same way that rats can be used, it was thought desirable to obtain some information concerning their vitamin B_1 metabolism. Two dogs

TABLE 3

The urinary excretion of vitamin B_1 by dogs

DATE	B_1 SUPPLEMENT	BASAL DIET: MODIFIED DIET NO. 123		
		Means of administering	Dog G daily output of B_1 in urine	Dog H daily output of B_1 in urine
	γ		γ	γ
5/ 2			40	40
5/ 3	....	.....	40	80
5/ 4			20	80
5/ 5	500	Subcutaneous injection	80	80
5/ 6	500	Subcutaneous injection	200	140
5/11 and 12	500	Subcutaneous injection	200	212
5/13	1000	Subcutaneous injection	350	350
5/14	1000	Subcutaneous injection	300	500
5/15	1000	Subcutaneous injection	500	440
5/16	1000	Subcutaneous injection	375	375
5/17	1000	Subcutaneous injection	300	600
5/18	2000	Subcutaneous injection	800	400
5/19	2000	Subcutaneous injection	900	500
5/20	2000	Subcutaneous injection	1000	800
5/21	2000	Fed by stomach tube	500	500
5/22	2000	Fed by stomach tube	800	400
5/23	2000	Fed by stomach tube	625	
5/24	2000	Fed by stomach tube	500	
5/25	2000	Fed by stomach tube	200	
5/26	2000	Fed by stomach tube	750	
5/27	2000	Fed by stomach tube	400	
5/28	2000	Enteric coated capsule	400	
5/29	2000	Enteric coated capsule	600	
5/30	2000	Enteric coated capsule	170	
5/31	2000	Enteric coated capsule	330	
6/ 1	5000	Subcutaneous injection	900	
6/ 3	5000	Subcutaneous injection	3000	
6/ 4	5000	Fed by stomach tube	1100	
6/ 5	5000	Fed by stomach tube	950	
6/ 6	5000	Fed by stomach tube	900	
6/ 7	5000	Enteric coated capsule	750	
6/ 8	5000	Enteric coated capsule	375	
6/ 9			900	
6/10			660	
6/11			200	
6/12			75	

(terrier type) weighing 9 to 10 kg. were placed in simple metabolism cages. The basal ration was a modification of diet 123 (Goldberger et al., '28) in which wheat gluten replaced the casein. This diet contains B_1 but was fed in regular amounts, thus keeping the B_1 intake from this source constant.

Table 3 shows the immediate response of the urinary B_1 to injected vitamin. The urinary excretion is nearly proportional to the amount injected. Only 40 to 60% of the injected vitamin was excreted in the urine as compared with 80% obtained with the rats. When the vitamin was fed by stomach tube the urinary output dropped to 20 to 30% of the vitamin administered. The B_1 found in the urine dropped still further when the vitamin was administered in the enteric coated capsules. Again, as with rats, cessation of the supplement caused a rapid drop in B_1 excretion.

DISCUSSION

Outstanding among the results described is the condition of excretion equilibrium. In every instance a constant vitamin B_1 diet eventually resulted in a constant vitamin output. This evidence is limited to a uniform environment because it is conceivable that exercise or other factors may disturb the equilibrium. The animal is considered to be in excretion equilibrium when the excreta have reached a maximum or minimum vitamin content on a uniform vitamin intake. That this is a true equilibrium may be seen from the fact that it may be approached from either direction.

It is probable that the idea of excretion equilibrium can be very useful in the further study of vitamin B_1 metabolism. An animal whose body has been severely drained of B_1 will obviously take time to reach excretion equilibrium at supraminimal levels. It may be assumed that some of the vitamin taken in is being stored in the body during this period which may be called the lag period. If we are justified in making this assumption it is to be expected that the duration of the lag period will vary depending on the magnitude of both the new feeding level and the body stores.

It is generally believed that there exists some optimal feeding level for each vitamin at and above which the animal will exhibit maximum health. Our data on excretion equilibrium indicate the possibility of determining this level. Shifting from low to high feeding levels it was discovered that no lag period could be observed at the 65 γ level and above. In other words, little or no further storage takes place once the animal has reached excretion equilibrium at some level between 40 and 65 γ. The problem of tissue storage can be attacked by analysis of the animal body, and a report of such a study is being prepared.

The distribution of the vitamin by an animal in excretion equilibrium deserves consideration. The fraction of the ingested vitamin found in the excreta of rats in excretion equilibrium at levels from 40 to 515 γ averages close to one-half. This in turn is about equally divided between urine and feces. Thus the distribution may be roughly described by the proportion: urinary B_1: fecal B_1: unaccounted for $B_1 = 1:1:2$. The explanation of this uniform distribution may lie in a fixed relation between the velocities of absorption, elimination (by urine), and destruction (utilization or detoxication). There is no evidence to indicate what may happen to the missing vitamin other than storage of a portion. Leong ('37) observed a failure of absorption at high levels (feeding an absorbate), and although we did not reach a similar level (feeding the pure vitamin) with rats, Schultz et al. ('38) observed a similar phenomenon with human subjects.

The quantitative determination of the vitamin B_1 excreted in the urine and feces appears of value for several purposes. The detection of severe cases of B_1 avitaminosis does not present a problem in medicine. On the other hand, many vague symptoms have been ascribed to slight B_1 deficiencies of long standing. A quick method of determining the state of B_1 metabolism should be useful in such cases. Furthermore, unusual conditions of absorption or metabolism such as might be produced in disease can be discerned and evaluated.

SUMMARY

The condition of vitamin B_1 excretion equilibrium is described for white rats.

Rats in excretion equilibrium on levels between 15 γ and 515 γ show the following distribution of the ingested vitamin: 25 to 35% excreted via urine, 20 to 30% via feces, and the remainder unaccounted for.

Animals with a previous history of low vitamin intake show a time lag before reaching excretion equilibrium. If the animal is brought to excretion equilibrium at a level between 50 to 65 γ a further increase in feeding level will show no time lag before reaching excretion equilibrium.

Subcutaneous injection of 200 to 500 γ per day (rats) caused the excretion via urine of 75 to 85% of the vitamin, whereas 10-kg. dogs showed urinary excretion of only 50% of 2000 γ injected.

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A STUDY OF THE BIOPHOTOMETER AS A MEANS OF MEASURING THE VITAMIN A STATUS OF HUMAN ADULTS

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It is now generally recognized that one of the early symptoms of vitamin A deficiency is reflected in subnormal dark adaptation.

Jeans, Blanchard and Zentmire ('37) were the first to report on the use of the biophotometer (superseding the Birch-Hirschfeld instrument used in their earlier work) as a simple clinical means for measuring the degree of dark adaptation of human subjects 6 years of age or older.

The success reported by Jeans and his co-workers in detection of vitamin A deficiency by the use of the biophotometer has been confirmed by Jeghers ('37), by Park ('36), and by Corlette et al. ('38). Palmer and Blumberg ('37), however, have recently used the biophotometer in a study of 585 elementary school children and have reported that repeated tests on the same children showed significant individual variations with a considerable improvement in the apparent dark adaptation as the young subjects became more familiar with the test. Maitra and Harris ('37) made a study of dark adaptation of school children with the Birch-Hirschfeld instrument and reported very consistent results with repeated tests on the same subjects.

Variations of light intensity in the Birch-Hirschfeld instrument are made by means of alterations in a diaphragm open-

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ing and in the position of a Goldberg wedge both interposed between the light source and the subject's eye. In the biophotometer the light intensity is varied by altering the resistance in series with a filament lamp which causes alterations in the temperature of the incandescent filament and results not only in changes of the light intensity but in the color of the light as well. Since the sensitivity of the retina (and of different areas of the retina) to light of different colors is not the same, it is desirable that the light source used in measurements of light thresholds include a constant portion of the visible spectrum and that the intensity factor alone be altered.

The work here reported represents a study of the specificity and reproducible qualities of measurements made with the biophotometer, and of factors that might affect these. The investigation was planned to determine: 1) the physiological range in the rate of dark adaptation of normal adults, 2) the dark adaptation response to increase of vitamin A intake of any subjects who appeared to have a subnormal rate of adaptation, 3) the day-to-day variations in rate of dark adaptation of individual subjects, and 4) the daily fluctuations in the measurements taken on individual subjects.

The brightness, in terms of microlamberts, of the central dot of the biophotometer quincunx (this dot is of 0.5 cm. diameter) corresponding to the different rheostat dial settings was calibrated by the National Bureau of Standards [2] in February, 1937, before any measurements were undertaken and again in November of the same year after all the measurements, reported here, had been completed.

The brightness values of the large diffusing plate (with the quincunx plate turned out of the line of sight) corresponding to different dial settings were measured with a Macbeth illuminometer. The value with the dial set at zero was arbitrarily taken as unity. A smooth curve was drawn through the actual measured values which furnished a curve of dial factors proportional to the brightness values of the large

[2] We are indebted to Ray P. Teele, associate physicist, National Bureau of Standards, for these calibrations.

diffusing plate for the different dial settings. The brightness of the central dot of the quincunx when the dial was set to read zero was then measured with the Macbeth illuminometer, which value multiplied by the several dial factors gave the calibration values furnished by the National Bureau of Standards. Each calibration was composited from the values obtained separately by three or four experienced persons. Repeated calibrations (closely following one another) showed no significant variation.

The instrument makers furnished a set of calibrations in terms of millifoot-candles of illumination in some plane, not specified, in front of the illuminated central dot of the quincunx. These calibrations [3] were made with a photronic (barrier-layer type) cell fitted with a visual correction color filter. The current output corresponding to the incident illuminations was converted into equivalent millifoot-candles of illumination by means of data supplied with the photronic cell.

The calibrations supplied by the manufacturers of the biophotometer, together with those supplied by the National Bureau of Standards, are shown in table 1.

The relationship between the brightness values for the illuminated central dot of the quincunx and the millifoot-candle values of illumination at a fixed distance from this dot should be constant. But inspection of the respective calibration values given in table 1 clearly shows that the millifoot candle values would have to be multiplied by a factor varying all the way from 3 to 6, depending upon the particular dial setting under consideration. This range of conversion factors applies, of course, to the particular biophotometer used. It would seem entirely evident, therefore, that the manufacturers' 'millifoot-candle' [4] values are not true indices of the threshold sensitivities of the subject's eyes.

[3] Personal communication from W. O. Frohring, designer of the biophotometer.

[4] The term millifoot-candle when used hereafter in this article to refer to the calibrations made by the photoelectric cell method with the fitted filter will be designated by quotations, 'millifoot-candle.'

The brightness of the large diffusing screen, used for bright light adaptation of the subjects, as determined by the National Bureau of Standards was 145 millilamberts. According to the calibration furnished by the manufacturers of the biophotometer, the illumination as transmitted through this bright diffusing screen was 1.1 foot-candles at the eye position.

TABLE 1

Calibration values for the brightness and intensity of illumination of the central dot of the quincunx corresponding to dial settings of the biophotometer

DIAL SETTING	BRIGHTNESS OF CENTRAL DOT MICROLAMBERTS [1]		ILLUMINATION OF THE CENTRAL DOT
	February, 1937	November, 1937	'm.f.c.' values furnished with instrument [2]
0	23.2	22.4	3.77
10	9.5	9.0	1.70
20	3.9	3.6	0.750
30	1.6	1.4	0.350
40	0.67	0.55	0.158
50	0.28	0.22	0.069
60	0.12	0.08	0.032
70	0.05	0.04	0.014
80	0.02	0.01	0.0064
90	0.008	0.006	0.0030
100	0.003	0.002	0.0013

[1] Calibration values furnished by the National Bureau of Standards.
[2] Values supplied by the manufacturers of the instrument.

EXPERIMENTAL

A series of light threshold measurements was made on each subject in accordance with the procedure previously described by Jeans, Blanchard and Zentmire ('37). The test period consisted of successive measurements of the thresholds of light sensitivity at 5-minute intervals during a 10-minute preliminary period of dark adaptation, immediately after a 3-minute period of adaptation to the bright light of the large diffusing screen, and at 3-minute intervals during a second dark adaptation period (the recovery period) of 9 minutes. The measurements are expressed in terms of microlamberts of brightness of the central dot of the quincunx and/or 'millifoot-

candles' of illumination at the eye position, the eye of the subject being directly in front of and at a distance of 30 cm. from the illuminated dots of the screen.

These series of measurements were made, in duplicate, for 106 adults (thirty-nine males and sixty-seven females), all of whom were employed at the Department of Agriculture. The subjects included persons engaged as clerical and professional workers. All subjects who were accustomed to wearing spectacles wore them throughout the test period.

Except for those subjects to whom halibut liver oil was later administered, the data presented are confined to the one measurement taken within an interval of approximately 20 seconds following the adaptation to the standard bright light or to this and the final measurement taken during the recovery period.

In the interest of brevity the threshold value measured during the 20-second interval immediately following the light adaptation period will be referred to here as the critical threshold measurement. It should be pointed out, however, that this critical threshold measurement is very difficult to make because of the exceedingly rapid rate at which the threshold value changes here. A few seconds' delay will give a significantly different reading. It is important, therefore, to verify this measurement by at least a second test; moreover, the interpretation of the critical threshold measurement should not be made apart from consideration of the final measurement of the 9-minute recovery period. Dark adaptation proceeds at a much slower rate during the latter part of the recovery period thus enabling the worker to obtain more reproducible measurements.

The frequency distribution of the critical light thresholds of the 106 subjects (grouped according to age and to threshold class intervals) is shown in tables 2 and 3.

The six subjects showing the highest critical threshold values and seven subjects having critical thresholds in the more central range of values were given approximately 5000 to 8500 international units of vitamin A per day in the form

of halibut liver oil over a period of 4 weeks. The data for two threshold values (one, the critical threshold value, and the other, the last measurement taken during the 9-minute

TABLE 2

Frequency distribution of critical threshold values for 106 adults of different age groups (microlambert intervals)

MICROLAMBERTS	AGE OF SUBJECTS (YEARS)				
	15–19	20–29	30–39	40–49	Over 50
0 –0.9	1	14	4	4	0
1.0–1.9	2	13	18	7	4
2.0–2.9	0	4	6	8	6
3.0–3.9	0	4	1	2	2
4.0–4.9	0	0	0	1[1]	0
5.0–5.9	0	0	0	0	0
6.0–6.9	0	0	0	0	1[1]
7.0–7.9	0	0	0	2[1, 2]	2[1]

[1] For critical threshold values of these subjects after administration of halibut liver oil, see table 4.

[2] One of these subjects (subject 73) is a case of hereditary and permanent hemeralopia, see text page 350.

TABLE 3

Frequency distribution of critical threshold values for 106 adults of different age groups ('millifoot-candle' intervals)

INTERVAL 'M.F.C.'	AGE OF SUBJECTS (YEARS)				
	15–19	20–29	30–39	40–49	Over 50
0 –0.09	0	0	1	0	0
0.10–0.19	0	7	2	3	0
0.20–0.29	3	13	6	3	0
0.30–0.39	0	4	11	5	4
0.40–0.49	0	6	8	4	3
0.50–0.59	0	1	0	5	2
0.60–0.69	0	4	1	1	2
0.70–0.79	0	0	0	0	0
0.80–0.89	0	0	0	1[1]	0
0.90–0.99	0	0	0	0	0
1.00–1.09	0	0	0	0	1[1]
1.10–1.19	0	0	0	1[2]	0
1.20–1.29	0	0	0	1[1]	2[1]

[1] For critical threshold measurements of these subjects after daily administration of halibut liver oil for a period of 4 weeks, see table 4.

[2] Subject 73, a case of hereditary hemeralopia, see text page 350.

recovery period) for the thirteen subjects to whom halibut liver oil was administered are shown in table 4. It is readily apparent from this table that these thirteen subjects naturally fall into two groups, eight (including the special case of subject no. 73) of whom showed no response to the halibut

TABLE 4

Threshold values of thirteen subjects before and after daily administration of halibut liver oil over a 4-week period

SUBJECTS		THRESHOLD VALUES BEFORE ADMINISTRATION OF HALIBUT LIVER OIL				THRESHOLD VALUES AFTER ADMINISTRATION OF HALIBUT LIVER OIL			
Type	Number	Microlamberts[1]		'Millifoot-candles'[2]		Microlamberts[1]		'Millifoot-candles'[2]	
		I[3]	II[4]	I[3]	II[4]	I[3]	II[4]	I[3]	II[4]
Normal	10	1.00	0.06	0.23	0.01	1.10	0.06	0.26	0.01
	19	1.45	0.11	0.36	0.02	1.50	0.14	0.38	0.03
	25	2.15	0.14	0.55	0.03	3.50	0.11	0.72	0.02
	20	2.40	0.22	0.59	0.05	4.00	0.14	0.79	0.03
	24	2.50	0.14	0.62	0.03	2.50	0.14	0.62	0.03
	39	2.70	0.25	0.63	0.06	2.20	0.18	0.53	0.04
	17	2.70	0.11	0.65	0.02	3.00	0.18	0.69	0.04
Abnormal	68	4.20	0.33	0.82	0.08	1.95	0.14	0.49	0.03
	72	6.40	0.80	1.07	0.18	4.00	0.25	0.77	0.06
	73[5]	7.00	0.96	1.18	0.22	6.40	1.45	1.09	0.36
	86	7.40	0.18	1.21	0.04	1.95	0.06	0.49	0.01
	71	7.40	0.33	1.23	0.08	1.65	0.14	0.42	0.03
	87	7.60	0.29	1.26	0.07	2.40	0.18	0.60	0.04

[1] Recorded dial readings converted into corresponding microlamberts of light brightness according to calibrations of the National Bureau of Standards.

[2] Recorded dial readings converted into corresponding 'millifoot-candles' according to the table supplied by the manufacturers.

[3] Critical threshold values (first reading after adaptation to the standard bright light).

[4] Last reading of the 9-minute recovery period following light adaptation.

[5] For discussion of case 73, see text, page 350.

liver oil administration as judged by either of the two measurements of threshold values and five of whom showed generally lower threshold values in both measurements. Every subject whose critical threshold value was lowered as a result of halibut liver oil therapy showed a significantly improved threshold at the end of the 9-minute recovery period. This also

confirms the view that these subjects were originally vitamin A deficient and were benefited by an increase in vitamin A intake.

The borderline regions for the critical light threshold and for the threshold at the end of the 9-minute recovery period, separating the normal from the vitamin A deficient subjects, were around 3.0 and 0.20 microlamberts, respectively, or according to thresholds of the 'millifoot-candle' values around 0.70 and 0.06, respectively. A very large number of subjects would need to be measured to establish the limits of the normal range by statistically acceptable methods. Such a range, if established, would need to be used with judgment since a subject with threshold values near the borderline might be a perfectly normal subject in the extreme end of the normal limit or a subject potentially capable of greater sensitivity to light but receiving insufficient vitamin A to support that.

Subject no. 73 (see table 4), who showed abnormally high light thresholds (for the critical light threshold and for the final light threshold measurement of the 9-minute recovery period) both before and after the 4-week period of vitamin A therapy, is a case of hereditary hemeralopia. This form of hemeralopia is not amenable to vitamin A therapy.

It is evident from the data given in tables 2 and 3 that the distribution within the group considered as normal subjects is fairly widespread. There is also a suggestion that for normal persons there is an increase in critical threshold values with increase in age. In a study of some 5000 school boys and some 6500 men employed in different industries, Collins and Britton ('24) found that the percentage of persons having a visual acuity of 20/20 (as tested by Snellen's test types) rises to a maximum at about 18 years of age and decreases rather rapidly thereafter. The measurement of the critical light threshold as made with the biophotometer is somewhat dependent upon visual acuity, so that the two measurements might be expected to have some relation to one another. No difference was observed between the critical threshold values for men and women.

Five adult subjects selected at random from within a group who showed approximately average threshold values or slightly lower were subjected to ten measurements at the same hour on 10 different days. The average difference between the maximum and minimum values for the ten measurements taken immediately following the light adaptation period was 0.99 ± 0.43 microlamberts. The average difference between the maximum and minimum values for this threshold in terms of the 'millifoot-candle' values was 0.25 ± 0.11. Repetition of the test did not show any consistent tendency toward lowered critical thresholds nor of lower thresholds at the end of the 9-minute recovery period which could be attributed to a factor of learning. In fact the averages of ten tests for any given subject were not significantly different from those of the first test.

Five subjects were tested three times during a single day at 9.00 A.M., 12.00 M (before lunch), and 4.00 P.M., and showed an average difference of 0.42 ± 0.21 microlamberts between the maximum and minimum critical threshold values. In terms of the 'millifoot-candle' values the average difference between the maximum and minimum values was 0.11 ± 0.06. From these data it does not seem probable that the time of day has any significant bearing on the measurements where normal subjects are concerned. It is, of course, not to be assumed that the same would be true of vitamin A-deficient subjects. Subsequent work has indicated that a vitamin A-deficient subject shows an improved dark adaptation when the measurement is made about 2 hours after the ingestion of food containing a significant amount of vitamin A—thereafter the dark adaptation power recedes.

DISCUSSION

The size of the central dot of the quincunx of the biophotometer is such that at 30 cm. distance it subtends a visual angle on the retina which would not exceed the area of the fovea. It would appear probable, therefore, that the cone structures of the retina as well as the rod structures re-

generate a substance (after bleaching) of which vitamin A is a component or that an extremely small number of rods was included in the area stimulated by the light from the central dot of the quincunx. It would seem more probable, however, that the image of the central dot of the quincunx is not formed directly on the fovea. This would be due to imperfect fixation of the eyes, an unconscious effort to place the image on a more sensitive (to dim light) area of the retina, namely, that part more highly populated with rod structures.

SUMMARY

Provided the worker is equipped with suitable means for frequent calibration of the intensity of the light emanating from the illuminated central dot of the test screen and from the large diffusing plate used for light adaptation, the biophotometer may be adequate for detecting marked dysadaptation in adults.

The method used by the National Bureau of Standards to calibrate the biophotometer used for the work reported here is described in the text.

Measurements of the critical threshold values (taken within a 20-second interval following the light adaptation period) are difficult to reproduce because of the extremely rapid decrease in the light threshold value at this time. It is, therefore, recommended that this measurement be verified for each subject. The threshold value at the end of the 9-minute recovery period is an important factor in the final interpretation of the test since it should reflect the same trend in threshold value as the measurement taken within the 20-second interval following the light adaptation period.

A critical threshold value (taken within a 20-second interval following the 3-minute light adaptation period) numerically greater than 3.0 microlamberts, together with a threshold value at the end of the 9-minute recovery period numerically greater than around 0.20 microlamberts, would appear to be fairly definite evidence of a subnormal power of dark adaptation in an adult. In terms of the calibrations furnished by

the manufacturers of the biophotometer these borderline thresholds would be on the order of 0.70 and 0.06 'millifoot-candles,' respectively. These borderline regions, however, probably are not sharply defined. There is at least a suggestion that these threshold values may be somewhat different for different adult age groups. Moreover, the normal range being quite widespread, a subject having a critical threshold value in the highest part of a so-called normal range might not have a sufficiently high level of vitamin A intake to maintain the lowest critical threshold of which he is potentially capable.

Five subjects with critical threshold values in the region of 4 to 8 microlamberts (or about 0.8 to 1.3 'millifoot-candles') showed definitely lowered thresholds, commensurate with normal critical thresholds, following daily administration of 5000 to 8500 international units of vitamin A in the form of halibut liver oil within a period of 1 to 2 weeks. The threshold values at the end of the 9-minute recovery period reflected the same trend of change.

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NICOTINIC ACID IN THE PREVENTION OF BLACKTONGUE OF DOGS[1]

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Elvehjem, Madden, Strong and Woolley ('37, '38) have reported that nicotinic acid and nicotinic acid amide will cure blacktongue of dogs and maintain them for 2 months. Street and Cowgill ('37) noted cure of mild symptoms of blacktongue in two dogs, and Chick, Macrae, Martin and Martin ('38) found a strikingly beneficial result from nicotinic acid in swine on a deficient diet.

It seemed desirable, therefore, to obtain further information in regard to the quantitative requirements of the dog for nicotinic acid.

EXPERIMENTAL

All of the experimental animals were placed on diet no. 123, the composition of which is given in table 1. Details of the handling of the animals have been given in publications by Goldberger and his associates ('28 a, b, '30). All of the experiments were run simultaneously.

Experiment I

Prevention of blacktongue with commercial nicotinic acid. Five dogs were placed on diet 123 and given semi-weekly doses of commercial nicotinic acid in aqueous solution by intramuscular injection. No attempt was made to select a dose

[1] Presented at the annual meeting of the American Institute of Nutrition, Baltimore, Maryland, on March 30, 1938.

on the basis of body weight since there is no information available on the factors governing the dog's requirement for nicotinic acid, or on the minimum amount necessary to prevent blacktongue. An arbitrary range of dosage was selected in an effort to given an amount both below and above the necessary minimum.

Dog 375, receiving 1 mg. of commercial nicotinic acid semi-weekly, developed blacktongue beginning on the twenty-fourth day of the experiment. The symptoms disappeared after the

TABLE 1

Composition of basic blacktongue-producing diet no. 123 [1]

ARTICLE OF DIET	QUANTITY	NUTRIENTS		
		Protein	Fat	Carbohydrate
	gm.	gm.	gm.	gm.
Corn meal [2]	400	33.6	18.8	296.0
Cowpeas (Vigna sinensis) [3]	50	10.7	0.7	30.4
Casein (purified) [4]	60	52.0	...	...
Sucrose	32	...	...	32.0
Cottonseed oil	30	...	30.0	...
Cod liver oil	15	...	15.0	...
Sodium chloride	10	...	...	...
Calcium carbonate	3	...	...	...
Total nutrients		96.3	64.5	358.4
Nutrients per 1000 calories		40.1	26.9	149.3

[1] The corn meal, cowpeas (previously coarsely ground), and salt are stirred into water and cooked in a double boiler of enamelware for about 1½ hours. Then the other ingredients are well stirred in, the total weight being brought to 2400 gm. with water (so that 1 gm. represents 1 calorie), and this finished mixture is served to the dog ad libitum.

[2] Whole maize meal (white) sifted as for human consumption.

[3] The variety known as the California black-eyed pea.

[4] Commercial casein leached for a week in daily changes of acidulated water, according to the method of McCollum, Simmonds, Shipley and Park ('22, Bull. Johns Hopkins Hosp., vol. 33, p. 398).

administration of 15 mg. of commercial nicotinic acid and the semi-weekly dose was then increased to 6.0 mg. The animal then went for 130 days without recurrence of blacktongue.

Dog 384, receiving 3.0 mg. of commercial nicotinic acid semi-weekly developed varying degrees of slight reddening of the buccal mucosa beginning on the forty-third day of the experiment. These lesions were never sufficiently characteristic to be diagnosed definitely as blacktongue. We have interpreted such lesions in the past as probably indicating a

marginal supply of the preventive factor. It is not unlikely that, had the dose been slightly less, a typical attack of blacktongue would have occurred.

Dog 387, receiving 25 mg. of commercial nicotinic acid semi-weekly, suddenly died on the eighty-ninth day of the experiment without having shown any sign of blacktongue. Degenerative changes in the central nervous system were found at autopsy. Diet 123 has been demonstrated to be deficient in lactoflavin, and since the pathological findings in this dog were similar to those in dogs previously reported from this laboratory ('33, '37, '38) on riboflavin deficient

TABLE 2

Prevention of blacktongue with nicotinic acid[1]

DOG NO.	WEIGHT AT BEGINNING OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	NICOTINIC ACID SEMI-WEEKLY	CALCULATED DAILY DOSE	REMARKS
	kg.	*kg.*	*mg.*	*mg.*	
375	6.2	6.4	1.0	0.28	First signs of blacktongue on 24th day. Receded with 15 mg. of nicotinic acid. No recurrence on 6.0 mg. bi-weekly for 130 days
384	6.4	7.2	3.0	0.85	Varying redness of buccal mucosa beginning on 43rd day. No definite symptoms of blacktongue
388	6.8	7.7	10.0	2.85	No signs of blacktongue
387	7.2	..	25.0	...	Dead on 89th day. Degenerative changes in the central nervous system. No signs of blacktongue
374	8.2	8.6	50.0	14.28	No signs of blacktongue

[1] Duration of experiment: 6 months. All animals on diet 123. Weekly supplement of 0.5 mg. of riboflavin given during last 3 months.

diets, the remaining animals in all the experiments were started on weekly doses of 0.5 mg. of riboflavin, intramuscularly. No other deaths occurred.

Dogs 388 and 374, receiving 10 mg. and 50 mg., respectively, of nicotinic acid semi-weekly, completed the 6-month experimental period without showing any signs of blacktongue.

Summary. One milligram of nicotinic acid semi-weekly was insufficient to prevent blacktongue in one dog; the dog on 3.0 mg. semi-weekly had symptoms suggestive of incipient blacktongue, and the dogs receiving 10 mg. and over semi-weekly had no signs of blacktongue.

Experiment II

The treatment of blacktongue and the prevention of recurrences with synthetic nicotinic acid. Five dogs were placed on diet 123 and allowed to develop blacktongue. After the symptoms of blacktongue became well advanced each animal was treated with a sufficient amount of synthetic nicotinic acid to cause all symptoms to recede. This was followed by a semi-weekly dose of 10 mg. of synthetic nicotinic acid for the remainder of the 6-month experimental period (132 days). The results are summarized in table 3.

The synthetic nicotinic acid was prepared from quinolinic acid.

TABLE 3

Treatment and prevention of recurrence of blacktongue with nicotinic acid[1]

DOG NO.	DAYS TO FIRST SIGNS OF BLACKTONGUE	TOTAL NICOTINIC ACID USED IN TREATMENT	SEMI-WEEKLY DOSE OF NICOTINIC ACID FOLLOWING RECOVERY	DAYS FROM START OF NICOTINIC ACID TO END OF EXPERIMENT	TOTAL NICOTINIC ACID GIVEN	WEIGHT, BEGINNING OF EXPERIMENT	WEIGHT, END OF EXPERIMENT
		mg.	*mg.*		*mg.*	*kg.*	*kg.*
365	34	50	10	139	430	8.4	7.9
389	29	59	10	142	439	5.1	6.8
392	31	80	10	141	460	3.3	7.1
393	24	75	10	146	455	4.4	4.6
396	24	76	10	143	456	5.2	5.8

[1] Duration of experiment: 6 months. All dogs on diet 123. Weekly supplement of 0.5 mg. of riboflavin given during last 3 months.

All five of the experimental animals developed symptoms of blacktongue which entirely disappeared under nicotinic acid treatment. There was no recurrence on semi-weekly doses of 10 mg. for 132 days.

All the animals received a weekly supplement of 0.5 mg. of riboflavin for the last 3 months of the experiment.

Experiment III

Treatment of blacktongue with nicotinic acid followed by treatment of recurrences. Five dogs were placed on diet 123 and allowed to develop blacktongue. When the symptoms became well established the animals were treated with enough commercial nicotinic acid in relatively small doses to cause the symptoms to subside. The administration of nicotinic

acid was then discontinued until the symptoms of blacktongue reappeared, when treatment was again instituted only until symptoms subsided. This procedure was repeated throughout the 6-month experimental period. The quantities given and the results are summarized in table 4.

Dog 395 died on the sixty-ninth day after having some slight reddening of the buccal mucosa and a bloody diarrhea. The symptoms were never characteristic of blacktongue. A peptic ulcer was found at autopsy.

The symptoms of blacktongue spontaneously receded in dog 348 and recurred after 55 more days. One dose of 20 mg. of commercial nicotinic acid was given and the symptoms receded. The animal completed the 6-month experimental period without receiving any additional nicotinic acid.

Dog 391 had six recurrences of acute symptoms in addition to the original attack during the 6-month experimental period. Each attack subsided following the administration of nicotinic acid. It was necessary to give a total of 278 mg. of commercial nicotinic acid to carry the animal through the experiment.

Dog 390 had three recurrences in addition to the original attack and received only 100 mg. of nicotinic acid during the 6-month experimental period.

Dog 394 had five recurrences in addition to the original attack and received a total of 200 mg. of commercial nicotinic acid during the 6-month experimental period.

This wide variation in the number of recurrences, in the time interval between recurrences, and in the quantity of nicotinic acid required strongly suggests that some factor other than body weight controls the dog's requirement for nicotinic acid.

Treatment of blacktongue with sodium nicotinate. One dog in an acute attack of blacktongue was successfully treated with 40 mg. of nicotinic acid in 2 days, given intramuscularly as sodium nicotinate.[2] Buccal lesions then recurred after an interval of 29 days and the administration of 30 mg. of nicotinic acid as sodium nicotinate again caused the acute lesions to recede, although the buccal mucosa remained slightly red for 35 days, when recurrence of acute symptoms again occurred.

A second animal in an acute attack of blacktongue was treated with a total of 70 mg. of nicotinic acid in 3 days, given

[2] The sodium nicotinate was prepared by dissolving nicotinic acid in distilled water and making the solution neutral to litmus with sodium bicarbonate.

TABLE 4

Treatment of blacktongue with nicotinic acid[1]

DOG NO.	RECURRENCE OF BLACKTONGUE	DAYS TO FIRST SIGN OF BLACKTONGUE	TOTAL NICOTINIC ACID GIVEN	DAYS FROM BEGINNING OF ONE ATTACK TO BEGINNING OF NEXT	WEIGHT AT BEGINNING OF EXPERIMENT	AVERAGE DAILY FOOD CONSUMPTION (6 MONTHS)	WEIGHT AT END OF EXPERIMENT	REMARKS
			mg.		*kg.*	*Calories*	*kg.*	
348		49	..	71	5.2	517	5.4	First attack spontaneously receded. Recovered.
	First	..	20	..				Received only 20 mg. nicotinic acid in 6 months
395	.....	..	..	..	..	328	..	Died 69th day. Peptic ulcer
391		15	40	22	11.6	587	7.5	Recovered
	First		40	31				Recovered
	Second		58	31				Recovered
	Third		20	16				Recovered
	Fourth		40	..				Mouth did not become entirely normal
	Fifth		40	..				Mouth did not become entirely normal
	Sixth		40	..				Mouth did not become entirely normal
390		52	40	31	6.0	587	5.3	Recovered
	First		20	44				Recovered
	Second		20	26				Recovered
	Third		20	..				Recovered
394		41	60	35	3.9	547	5.4	Young dog. Recovered
	First		20	25				Recovered
	Second		30	21				Recovered
	Third		40	31				Recovered
	Fourth		40	21				Recovered
	Fifth		10	..				Symptoms subsiding at end of experiment

[1] Duration of experiment: 6 months. All dogs on diet 123. Weekly supplement of 0.5 mg. of riboflavin given during last 3 months.

intramuscularly, as sodium nicotinate. The acute symptoms subsided but the buccal mucosa showed slight varying degrees of redness for 52 days, at which time a recurrence of acute symptoms of blacktongue occurred. Following one dose of 20 mg. of nicotinic acid intramuscularly, the buccal lesions completely subsided and the next recurrence of symptoms started 10 days later.

CONCLUSIONS

1. Recession of acute symptoms of blacktongue followed the administration of either sodium nicotinate or nicotinic acid.
2. The total quantity of nicotinic acid necessary to cause the acute symptoms of blacktongue to subside appears to be from 20 mg. to 60 mg.
3. The wide variation in the amount of nicotinic acid required to prevent recurrence of the acute symptoms of blacktongue suggests that some factor other than body weight may be involved.
4. A semi-weekly dose of 3.0 mg. of nicotinic acid appears to be barely sufficient to prevent blacktongue under the conditions of this experiment. A semi-weekly dose of 10 mg. of nicotinic acid was ample to prevent blacktongue for a period of 6 months.

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THE CONTENT OF REDUCED ASCORBIC ACID IN BLOOD PLASMA IN INFANTS, ESPECIALLY AT BIRTH AND IN THE FIRST DAYS OF LIFE

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ONE FIGURE

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In previous studies ('37, I and II) it was found that neither the capillary fragility as determined by a constricting arm-band test nor the content of reduced ascorbic acid in serum, determined on cord blood, gave any evidence of latent scurvy at birth. On the other hand, 26% of the 10-day-old infants studied showed low capillary resistance, as did 50% of infants from 1 to 6 months old, fed exclusively on human milk. A decrease in the ascorbic acid in the blood during the first few weeks of life was suggested, but not proved with the method available at that time (Lund and Lieck, '36), which required 1 to 5 ml. of serum for analysis. The present study represents further investigation into this problem, and also some studies of the normal values for reduced ascorbic acid in blood plasma during infancy.

In this country Abt, Farmer and Epstein ('36) have given data for reduced ascorbic acid in blood plasma of children 'on an adequate vitamin-C dietary' which includes orange juice daily for infants over 6 weeks of age. Cord blood, in five determinations averaged 1.02 mg. per 100 ml., the value being identical with that of the venous blood of the mother

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in each case. The average value for six infants from 3 to 6 days old was 1.12 mg. per 100 ml., and for ten infants from 6 to 26 weeks old, 1.34 mg. per 100 ml. Values averaging 1.19 mg. per 100 ml. were given by eleven adults, considered normal. These authors characterize 0.6 to 0.7 mg. per 100 ml. as subnormal. Greenberg, Rinehart and Phatak ('36) observed that the plasma ascorbic acid of adults varied with the intake of vitamin C. The ascorbic acid content of plasma of eleven subjects whose diets were low in vitamin C averaged 0.55 mg. per 100 ml., that of nine subjects whose diets were rated fair, 0.75 mg. per 100 ml., while eight subjects rated as ingesting good diets showed an average level of 1.1 mg. of ascorbic acid per 100 ml. of plasma.

TABLE 1

Accuracy of method. Titration of pure ascorbic acid with dichlorophenol-indophenol

Strength of ascorbic acid solution[1]	*Titer of dye solution for 0.2 ml. of ascorbic acid solution*	
mg. per 100 ml.	observed ml.	calculated ml.
0.5	0.049	0.050
	0.049	
0.33	0.032	0.033
	0.033	
0.25	0.024	0.025
	0.025	
0.20	0.020	0.020
	0.020	

[1] Prepared by dilution of 0.01% solution in 2.5% metaphosphoric acid.

The analyses reported here were carried out in February and March of 1937. The micro-method of Farmer and Abt ('36) was used exactly as described. Metaphosphoric acid was added to the blood within 30 minutes after the sample was taken, so no special measures were taken to prevent oxidation. Blood was taken from the heel of infants, from digital pulp of adults. The accuracy of the analysis (tables 1 and 2) seems satisfactory.

The results are given in tables 3 to 6. The values for plasma ascorbic acid of women immediately after delivery

were unexpectedly low, averaging 0.26 mg. per 100 ml. (table 3). As these women had been in the hospital for periods varying from 3 days to 4 weeks before delivery, no general classification of intake of ascorbic acid is possible. It may be assumed that the diets throughout pregnancy were probably low in vitamin C as few of the women habitually ate fresh fruit. Subjects nos. 15, 18 and 23, however, were exceptions to this rule, as each had ingested citrus fruit daily throughout pregnancy. The plasma ascorbic acid

TABLE 2

Recovery of ascorbic acid added to plasma

	Plasma alone	*Plasma plus ascorbic acid solution* [1]	*Difference observed*	*Theoretical difference*
Plasma I	0.012	0.036	0.024	0.0245
	0.012	0.037	0.025	
	0.014	0.039	0.025	
	0.012	0.036	0.024	
	0.016	0.037	0.021	
	0.017	0.040	0.023	
Plasma II	0.025	0.049	0.024	0.023
	0.028	0.051	0.023	
	0.026	0.050	0.024	
	0.029	0.054	0.025	
	0.028	0.051	0.023	

[1] 1 ml. of ascorbic acid solution (10 mg. per 100 ml.) added to 9 ml. plasma. Figures represent milliliters indophenol solution without subtraction of blank.

values for these women were within the range observed for the group as a whole. To secure further information as to whether these low values for plasma ascorbic acid were due primarily to the drain of pregnancy or to dietary habits, values were also obtained from three groups of non-pregnant women. Two of the groups, patients in the medical wards, and nurses, received the same diet as the pregnant women while in the hospital; the third group consisted of healthy women whose diets habitually included citrus fruits. It will be observed (table 4) that the sick women showed values for plasma ascorbic acid comparable to those of the parturient

women. Healthy women ingesting the same diet (column 2, table 4) maintained a plasma ascorbic acid averaging 44% higher than that of the sick and parturient women, but less than half the average value found in the women ingesting larger amounts of ascorbic acid. These studies thus do not exclude the effect of diet entirely, but the fact that the values for the three pregnant women whose diets were high in

TABLE 3

Reduced plasma ascorbic acid of normal women immediately after delivery, of cord blood, and of normal infants at birth and during the first 10 days of life

SUBJECT NO.	MOTHER	REDUCED PLASMA ASCORBIC ACID, MILLIGRAMS PER 100 CC.					CAPILLARY FRAGILITY [1] NUMBER OF PETECHIAE	
		Cord blood	Baby at birth	22 to 26 hours	5th day	10th day	2nd day	10th day
1	0.72	2.60	1.24		0.12	0.16		6
2	0.36	0.76	0.44		0.24	0.24		40
3	0.48	1.56	0.56		0.12	0.16		22
4	0.24	0.36	0.48		0.12	0.12		2
5	0.20	0.84	0.48		0.28	0.28		5
6	0.28	1.08	1.00		0.12	0.08		2
7	0.60	1.16	1.16		0.56	0.36		20
8	0.24	1.16	0.76		0.36	0.40		>100
9	0.20	1.16	0.76		0.32	0.16		1
10	0.40	1.24	0.92		0.48	0.52		4
11 [2]	0.12	0.72	0.28		0.16	0.16		2
12 [2]		0.72	0.28		0.12	0.16		3
13	0.28	0.68	0.80	0.44		0.28	1	>100
14	0.12	0.68	0.72	0.48		0.32	0	18
15	0.40	2.04	1.28	0.64		0.64	0	3
16	0.08	0.52	0.40	0.36		0.32	0	1
17	0.16	0.80	0.56	0.28		0.24	0	14
18	0.20	1.12	0.96	0.40		0.24	0	0
19	0.16	0.96	0.60	0.36		0.24	0	37
20	0.20	1.16	0.76	0.28		0.12	1	>100
21	0.12	0.56	0.52	0.28		0.20	0	5
22	0.16	0.52	0.36	0.36		0.24	0	0
23	0.20	0.76	0.56	0.36		0.52	0	0
Average	0.26	1.07	0.69	0.39	0.25	0.27		

[1] Capillary fragility at different ages is given as the total number of petechiae on both arms in an area in plica cubiti, 4 cm. in diameter, after stasis on the upper arm for 4 minutes with a pressure of 70 mm. mercury. Over 10 petechiae are considered to indicate lowered capillary resistance.

[2] Numbers 11 and 12 are twins.

vitamin C were as low as the average value for their group and far lower than the values observed in non-pregnant women with similar intakes may be considered evidence of a definite tendency to low values for plasma ascorbic acid in late pregnancy. This finding is in line with former experience (Braestrup, '37, II); the addition of 100 mg. of ascorbic acid daily to the diet of pregnant women caused only moderate increases in the serum ascorbic acid.

The first three columns in table 3 show an interesting relation between the blood values of the mothers, of the um-

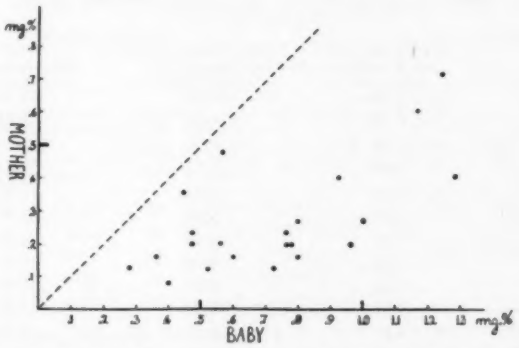

Fig. 1 Graph showing the percentage of ascorbic acid in the blood of the newly born babies as compared to the values for the mothers.

bilical cords and of the infants at birth (the samples were taken within 30 minutes after birth). The difference between the values for the mothers and for the cord blood has been demonstrated in a previous study ('37, II); as pointed out, the difference is probably caused by a concentration of ascorbic acid in the placenta. The difference between the values for the mothers and for the baby also supports this hypothesis, as figure 1 shows that all the infants have higher blood values than their mothers. There is a tendency for a relatively high value in the mother to be accompanied by a relatively high value in the baby.

The ascorbic acid content of the cord blood is almost constantly higher than that of the heel blood from the newly-born child (table 2, columns 2 and 3). This difference does not seem to be adequately explained by the way in which the blood samples are obtained, as no such difference could be demonstrated by simultaneous examinations of venous blood and capillary blood from the digital pulp in five normal adults. To bring the hypothesis that ascorbic acid is concentrated in the placenta into agreement with this observa-

TABLE 4

Reduced plasma ascorbic acid in non-pregnant women

		Healthy women	
	Patients medical ward, milligrams per 100 ml.	Citrus fruits twice a week or less, milligrams per 100 ml.	Citrus fruits daily, milligrams per 100 ml.
	0.16	0.24	0.72
	0.24	0.16	0.96
	0.28	0.24	0.88
	0.40	0.24	0.88
	0.20	0.40	1.04
	0.20	0.78	1.32
	0.12	0.36	0.72
	0.12	0.24	0.56
	0.68	0.24	0.48
	0.10	0.24	1.02
		0.48	
		0.38	
		0.64	
Average:	0.25	0.36	0.86

tion, a considerable loss of ascorbic acid in the fetus must be assumed. The concentration of ascorbic acid in the circulation of the fetus will then always be lower than that in the supply from the placenta through the cord vein (from which practically all the blood obtained for analysis comes). That such a loss is likely to take place is indicated by the increased demand for vitamin C during pregnancy, and also by the very marked loss of ascorbic acid in the first short period of extrauterine life as shown by the descrease of nearly 50% in plasma ascorbic acid during the first 24 hours and the

further decrease before the baby is 5 days old (table 3, columns 4 and 5). A decrease in this period could certainly be expected, as the vitamin C content in the milk of the first days is very low (Kasahara and Kawashima, '36, I; Correns, '37) and only small quantities are consumed. But even if there were no intake at all of vitamin C during the first hours, the decrease is remarkable as compared to the very moderate difference between the afternoon values and the fasting values in the morning, 24 hours after the last intake of vitamin C, observed in the older infants (table 6).

TABLE 5

Normal infants fed boiled cow's milk, examined before and during administration of pure ascorbic acid

SUBJECT NO.	AGE, WEEKS	WEIGHT, GRAMS	ASCORBIC ACID DAILY, MILLIGRAMS	PLASMA ASCORBIC ACID MILLIGRAMS PER 100 CC.						CAPILLARY FRAGILITY [1] NUMBER OF PETECHIAE
	Before administration			Before administration	5th day	10th day	15th day	23rd day	28th day	
24	4	3300	20	0.24	0.36	0.24	0.56	0.56	0.56	8
25	5	4100	20	0.34	0.44	0.60	0.76	0.72	0.68	6
26	4	3275	20	0.24	0.48	0.48	0.60	0.52	0.60	3
27	3½	4035	10	0.12	0.20	0.28	0.20			6
28	2	4015	10	0.16	0.28	0.32	0.28			1

[1] Capillary fragility was determined on the same days as the blood analyses were carried out; the figure given is the maximum number of petechiae obtained in an individual examination (see legend of table 1).

As seen in table 5 the low plasma ascorbic acid level remains fairly constant, at least for 4 to 5 weeks when the infant is fed cow's milk. The addition of vitamin C causes an increase for 10 to 15 days after which a fairly constant level is maintained according to the dose administered. Whether the ascorbic acid is given in pure form or in the form of orange juice containing the same amount seems to be immaterial (tables 5 and 6). Table 5 shows further a definite difference in the plasma ascorbic acid when 10 and 20 mg. ascorbic acid, respectively, are given daily. This is of considerable practical significance, as about 20 mg. of ascorbic acid—3 to 5 mg. per kilogram body weight—represents the

minimum daily amount an infant can be expected to get when fed exclusively on human milk, at least in summer and autumn. The vitamin C content of human milk in the first months of lactation is from 2.2 to 6 mg. of ascorbic acid per 100 ml. (Harris and Ray ('35) 6 mg. per 100 ml.; Schiaparelli and Buogo ('35) 2.2 to 4 mg. per 100 ml.; Selleg and King ('36) average about 5.5 mg. per 100 ml. and, after administration of orange juice, several values over 8 mg. per 100 ml.; Wachholder ('36) Ferdinand ('36) and Correns ('37) 2.7 to 5.6 mg. per 100 ml.; Kasahara and Kawashima ('36 II) 3.5 to 6 mg.

TABLE 6

Normal infants fed boiled cow's milk and orange juice

SUBJECT NO.	AGE, WEEKS	WEIGHT, GRAMS	DAILY AMOUNT OF ORANGE JUICE	PLASMA ASCORBIC ACID MILLIGRAMS PER 100 ML.					CAPILLARY FRAGILITY[1] NUMBER OF PETECHIAE
				Fasting	5 to 7 hours after orange juice			Average	
			ounces						
29	11	5075–5900	1	0.56	0.60	0.80		0.65	8
30	10	4600–5370	1	0.68	0.88	0.88		0.81	7
31	9	4576–5610	1	0.72	0.64	0.52	0.56	0.61	24
32	8	5000–5675	1	0.60	0.60	0.60	0.68	0.62	20
33	22	7375–8200	2	0.56	0.92	0.76	0.64	0.72	4
34	21	6235–7025	2	0.68	0.68	0.56		0.64	4
35	9	5075–6265	1		0.72	0.76	0.76	0.75	4
		Average		0.63		0.70			

[1] Capillary fragility as in table 5. In subjects nos. 31 and 32, three out of five and two out of four examinations respectively, showed more than a total of 10 petechiae.

per 100 ml.). An everage daily intake of 150 ml. milk per kilogram body weight allows a minimum of 3 mg. and in most cases a customary intake of 5 to 8 mg. per kilogram of ascorbic acid daily for the first few months. Until reliable methods for the determination of vitamin C subnutrition are available this must be considered to be the standard intake. In practice this means that all infants who are not given human milk must have an extra supply of vitamin C corresponding in the first few months to at least 20 mg. of ascorbic acid daily. As the vitamin C content of human milk varies so greatly with the

diet, it is preferable to give extra vitamin C in winter and spring, even to infants fed on human milk. Further investigations into this problem are in progress.

Results of the examinations for capillary fragility are also given in the tables. The figures in table 2 are almost identical with previous observations in Copenhagen in the summer of 1936 (Braestrup, '37, I). It can be seen that if the newly born and the 10-day-old children, respectively, are considered as groups, the capillary fragility follows the plasma ascorbic acid very closely; but no relation whatsoever between the two can be demonstrated in the individual case. This confirms the findings of Abt, Farmer and Epstein ('36) and is in line with the latest observation that the capillary permeability is not regulated by ascorbic acid but by another factor, P, now isolated as 'citrin' (Armentano, Bentsáth, Béres, Rusznyák and Szent-Györgi, '36). My previous observation ('37, I) that the effect of pure ascorbic acid on capillary fragility, if any, is slower than that of tomato juice, is also in agreement with this. It must be pointed out, however, that the two cases of low capillary resistance in table 5 (31 and 32) cannot be due to lack of vitamin P, as both these infants had received orange juice for a long period; a slight rash (sudamina) in one of the children might have caused the low resistance, but in the other case no such factor was demonstrated.

SUMMARY

Determinations of reduced plasma ascorbic acid (Farmer and Abt, '36) in February and March show:

Twenty-two women averaged 0.26 mg. per 100 ml. at the end of their pregnancy; the values of the infants just after birth were higher (average 0.69 mg. per 100 ml.) and still larger amounts were present in cord blood (1.07 mg. per 100 ml.).

During the first 24 hours after birth the plasma ascorbic acid decreases nearly 50%, and when the infant is 10 days old the average value found is 0.27 mg. per 100 ml. which is almost the same value as was found in the maternal blood (0.26 mg. per 100 ml.). When cow's milk is given the plasma ascorbic

acid remains at or slightly below this value for at least 4 to 5 weeks. The addition of 20 mg. of ascorbic acid or 1 ounce of orange juice daily is followed by an increase to from 0.56 to 0.81 mg. per 100 ml. whereas 10 mg. daily gave a considerably smaller increase in two cases.

Capillary resistance is very high (normal) in all cases at birth, but descreased in 39% of twenty-three normal 10-day-old infants. Two out of twelve normal older infants had a low capillary resistance in spite of a daily intake of orange juice.

The author wishes to express his gratitude to Dr. E. D. Plass for his kind permission to carry out the examinations of the women and the newly born children in the department of obstetrics.

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THE CHANGE IN TOTAL ENERGY METABOLISM OF RATS RECEIVING A DIET DEFICIENT IN INORGANIC CONSTITUENTS [1]

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In a previous study (Kriss and Smith, '37) it was observed that when young rats are kept on a diet deficient in mineral salts their basal metabolism becomes markedly increased in comparison with the basal metabolic rate of rats receiving an adequate supply of salts. While this is in harmony with the observed facts regarding the relative efficiency with which these animals utilize their respective diets, an intimately related and obviously important question is how the total energy metabolism is changed as a result of the mineral deficiency. The experiments recorded in this paper contribute to the elucidation of this question.

EXPERIMENTAL PART

The same rats were used in this investigation as were employed in the previous study of the fasting metabolism (Kriss and Smith, '37). The preparation of the animals for the experiment was as follows: at the age of 21 days the rats were placed on an adequate stock diet and allowed food ad

[1] The data reported in this paper were taken from a dissertation presented by Max Kriss in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1936.

A preliminary report was presented before the American Institute of Nutrition, third meeting, Washington, March, 1936.

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libitum. When they reached a weight of approximately 120 gm., food was withdrawn for 24 hours after which the animals were subjected to an 8-hour metabolism test. After the completion of this test they were given the synthetic diets by pairs. One of the pair received diet I, which was extremely low in minerals; this food was composed of specially washed casein, 18%; dextrin, 55%; and hydrogenated fat, 27%. The other rat, a litter mate, which served as the control, received the same diet plus 4% of salt mixture (Osborne and Mendel, '19). This ration containing the mineral salts is designated diet II. All rats received the same vitamin supplements, which consisted of dried yeast (200 mg. per day), alcoholic extract of wheat germ (1 cc. per day, equivalent to 2 gm. of wheat embryo) and 6 drops of cod liver oil per day. The analysis of individual dietary constituents and of the mixed diet is given in table 2.

The paired feeding technic was used; food was offered twice daily in two equal portions, one given about 8.00 A.M. and the other at about 6.00 P.M. and the animals were conditioned to consume their food promptly. The rats were kept on the experimental diets for a period of approximately 3 months. During this period determinations of the total respiratory metabolism were made, with a few exceptions, at approximately monthly intervals, as indicated in the schedule of experiments (table 1), the various intervals being referred to, respectively, as period 2, period 3 and period 4. Few changes in the allowance of food were made; none during the week preceeding any of the respiration tests. The daily food intake of the animals in the different experimental periods are shown in table 3. In period 2 the same quantities of food were consumed by the animals during the last 12 days prior to the respiration tests. In periods 3 and 4 the rations were kept constant for much longer periods.

A necessary requirement in this part of the investigation was that the animals consume their food promptly so as to make possible the inclusion of the entire effect of the food eaten in the measurement of the respiratory metabolism. For

this reason, the rations do not represent the full capacity of the animals to eat, not even of the salt-deficient animals. The limitation of the food supply was, however, not entirely arbitrary. It was made for each pair of rats on the basis of their response to the food offered which was determined by close observation of the animals. An early attempt to feed the animals to capacity resulted in an early loss of one of the low-salt animals (rat 1).

In none of the periods were the rations less than required for maintenance. In period 2 the rations were decidedly supermaintenance, since they permitted fair growth. The

TABLE 1

Schedule of experiments

RAT NOS.		PERIOD 2		PERIOD 3		PERIOD 4	
Low salt	Control	Age in days	Days on diet	Age in days	Days on diet	Age in days	Days on diet
1[1]	2	66	31				
3	4	68 and 67	32 and 31	98	62	122	86
6	7	67	33	90	56	122	88
9	10	72	35 and 34	97	60 and 59	124	87 and 86
11	12	76	38 and 39	101	63 and 64	128	90 and 91
13[2]	14	63	31	93	61		
19	20	60	27	90	57	117	84
23	24					118	82
25	26					121	82

[1] Rat 1 died 12/21/34 as a result of salt deficiency.
[2] Rat 13 died 2/9/35 as a result of salt deficiency.

exact time that the morning meal was fed prior to a respiration test, and the time that was required by the animals to consume it, were always noted. In most cases these intervals ranged from about 20 to 30 minutes.

All measurements of the total respiratory metabolism of the rats were started very soon after the morning meal was eaten and continued for 8 consecutive hours, these measurements being made simultaneously with both pair mates. The respiration apparatus used and the conditions of temperature, ventilation and light have been described by Kriss and Smith ('37). As in the previous study close observation was

made of the activity of the animals while in the respiration chamber.

In connection with the present investigation of the total energy metabolism a study was made of the effect of the mineral deficiency on the oxidation of protein, fat and carbohydrates, as well as on the specific dynamic action of the diet. This part, however, will be discussed in a subsequent paper (Kriss and Smith, '38).

TABLE 2

Analysis of individual dietary constituents and of the mixed diets

SUBSTANCE	MOISTURE	ASH	NITROGEN	CARBON	ETHER EXTRACT
	%	%	%	%	%
Casein	8.9	0.052	13.82	50.51	0.29
Dextrin	6.93	0.120	0.28	38.26	0.30
Crisco	0.07	..	0.13	73.99	
Diet I (low-salt)					
as determined			2.56	49.31	
as calculated [1]	4.53	0.075	2.67	50.09	27.20
Diet II (control)					
as determined			2.46	47.64	
as calculated [1]	4.35	4.11	2.57	48.16	26.15
Daily dose of	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Yeast	9	20	15	77	3
Wheat germ extract	787	5	5	168	8
C.L.O.	..	..	0	111	155

[1] On the basis of the analyses of the individual components.

RESULTS

The influence of activity

It was shown, in a previous paper (Kriss and Smith, '37), that the mineral deficiency affects both the activity and the metabolic rate of fasting rats. In this condition rats which received the low-salt diet are less active than their controls. When the fasting metabolism is reduced to a basis of comparable size and inactivity, it is considerably higher for the low-salt animals than for the controls. It is obviously important to ascertain whether or not similar differences prevail in the feeding periods as during fasting. For this purpose

we have followed the method previously applied to the fasting data, and have expressed the total metabolism also on two different bases: 1) including hours of activity and, 2) excluding hours of activity. These results are set forth, for the different periods, in table 3.

The number of hours during which the rats were visibly inactive varied with the diet in the different periods. Invariably the rats receiving the low-salt diet were less active than were their controls. In some cases, especially with the low-salt rats, as many as 6 or 7 hours represented hours of inactivity. In others only 4 or 5 hours could be thus considered. In two cases (rat 20, period 2 and 3) the hours of inactivity numbered 3 and in only one case (rat 4, period 2) the hours of inactivity were limited to 2. With one exception (rats 13 and 14, period 3), it was possible to average a greater number of hours of inactivity for the low-salt animals than for the controls.

The justification for averaging determinations of the heat production, in the feeding periods, representing different numbers of hours of inactivity, lies in the fact first demonstrated by Kriss, Forbes and Miller ('34) and confirmed by our observations in the present investigation, that, under our conditions of experimentation, the metabolism after the ingestion of food, when unaffected by irregularities due to activity, is not curvilinear, but is virtually on a level.

The results in table 3 show that the differences in the heat production due to activity are, as in the fasting periods previously discussed by Kriss and Smith ('37), considerably smaller with the animals which received the low-salt diet than with the controls. In view of the consistent character of the results in all the experimental periods the significance of the data seems to be unquestionable.

Obviously, the mineral deficiency depressed the activity of the animals in the feeding periods as it did during fasting. This confirms the observation of Smith and Smith ('34). The total effect, however, of the activity of either the low-salt animals or of the controls on the average hourly heat production, is relatively small.

TABLE 8

Influence of the low-salt diet on the activity and the total energy metabolism

RAT NO.	DIET NO.	PERIOD 2				PERIOD 3				PERIOD 4			
		Daily food intake [1]	Body weight	Average hourly heat production		Daily food intake [1]	Body weight	Average hourly heat production		Daily food intake [1]	Body weight	Average hourly heat production	
				Including hours of activity	Excluding hours of activity			Including hours of activity	Excluding hours of activity			Including hours of activity	Excluding hours of activity
		gm.	*gm.*	*cal.*	*cal.*	*gm.*	*gm.*	*cal.*	*cal.*	*gm.*	*gm.*	*cal.*	*cal.*
1	I	4.0	140	823	813								
2	II	4.2	155	834	795								
3	I	4.0	140	832	734	4.0	155	860	794	4.0	155	842	758
4	II	4.2	147	781	712	4.2	154	819	708	4.2	146	796	743
6	I	4.0	141	813	806	4.6	145	784	783	4.6	154	784	768
7	II	4.2	161	810	745	4.8	167	769	677	4.8	173	759	702
9	I	5.0	154	975	952	5.0	166	1020	1011	5.0	165	1122	1103
10	II	5.2	168	975	794	5.2	165	919	826	5.2	168	861	749
11	I	6.0	157	989	953	5.0	159	933	899	5.0	164	935	886
12	II	6.2	183	960	895	5.2	191	919	818	5.2	201	900	885
13	I	6.0	165	1046	989	5.0	179	936	872				
14	II	6.2	185	1025	959	5.2	189	866	840				
19	I	4.0	136	818	800	4.0	147	761	703	4.0	158	830	829
20	II	4.2	146	968	814	4.2	152	870	689	4.2	173	948	
23	I									4.0	138	816	794
24	II									4.2	164	811	707
25	I									4.0	155	832	848
26	II									4.2	164	732	695
Ave.	I		148	899	864		159	882	844		156	880	855
Ave.	II		164	908	816		168	860	760		170	830	747

[1] The vitamin supplements, which were the same for all rats in all periods, amounting to 559 mg. of moisture-free substance per day, are not included in the quantities of food given in this table.

The influence of the mineral deficiency on the total energy metabolism

The influence exerted by the mineral deficiency on the total metabolism is shown in table 3. No attempt was made to reduce the total metabolism to a basis of uniform weight or size. With the basal metabolism changing from period to period (Kriss and Smith, '37), because of advancing age, retarded growth or lack of mineral salts in the diet, no defensible method seems to be available for calculating the results on total metabolism either per unit of body weight or surface. The following comparisons are therefore made with the data unaltered on account of differences in body weight.

In period 2 the average hourly heat production, excluding hours of activity, is with one exception (rat 19) higher for the low-salt rats than for the controls, although the body weight of the latter is, without a single exception, higher. The average body weight (148 gm.) of the low-salt animal is, in this period, 10% less than the average weight (164 gm.) of the controls. Nevertheless, the average value for heat production (864 calories) of the salt-deficient animals is 6% higher than the average value (816 calories) for the controls. Similar effects of the mineral deficiency on the metabolism are observed in periods 3 and 4.

The body weights of the control animals in period 3 are in all cases but two (rats 3 and 9) higher than those for the low-salt animals, while without a single exception the heat production of the latter group is higher. The average value (844 calories) representing the hourly metabolism (uninfluenced by activity) of the low-salt animals exceeds the value (760 calories) for the controls by 11%, although the average body weight of these low-salt animals is 5% less than that of the controls.

In period 4 the hourly heat production of the low-salt animals also exceeds that of the controls, with one exception. The average hourly heat production (855 calories) for the low-salt animals is 14% higher than the value (747 calories) for the controls, although the average body weight (156 gm.)

per day, are not included in the quantities of food given in this table.

of the low-salt animals is 8% less than that (170 gm.) of the controls.

In all the foregoing comparisons of the heat production, the influence of activity was eliminated. If the comparisons are made on the basis of the data for heat production, including hours of activity, the effects of the mineral deficiency appear to be less marked, but in the same direction, especially if the differences in the body weight are also taken into consideration. A consideration of the foregoing data leads to the obvious conclusion that the mineral deficiency caused an increase in the total metabolism.

It is of interest to compare the increases in the total metabolism with the increases previously noted (Kriss and Smith, '37) in the basal metabolism. This can be best accomplished by comparing the results obtained in period 4 (table 3) with results obtained with the same animals during the final fasting period. These two periods were only 1 day apart, hence changes in the body weights between these periods can be disregarded.

During fasting the hourly heat production of the mineral deficient rat 3 exceeded the heat production of his control by 15% whereas in the feeding period the difference (compare 758 with 743 calories) is only 2%. The excess heat production of rat 6 (low-salt) over that of rat 7 (control) is the same (9%) in the feeding as it was in the fasting period. With rats 11 and 12 the difference was 7% during fasting, while there is no difference in the feeding period. The total metabolism of rat 23 (low-salt; 794 calories) is greater than the metabolism of rat 24 (707 calories) by 11%, whereas during fasting the difference was 16%. With rats 25 and 26, however, the difference in the fasting period (11%) was less than the difference (15%) in the feeding period. The average difference in heat production between the animals which have just been compared is 7.4%, in favor of the low-salt animals, in the feeding periods, and 10.2% in the fasting periods. No comparison can be made with rats 9 and 10, because of the abnormal condition of rat 9.

The foregoing comparisons indicate that the influence of the mineral deficiency on the metabolism was somewhat enhanced during the fasting periods, as compared with the feeding periods, and that the increases in the total metabolism can be accounted for largely by increases in the basal metabolism.

SUMMARY

Young rats fed a diet defective in inorganic salts for about 3 months showed the following differences in the total metabolism, as compared with their calorie controls, whose diet contained 4% of salts:

After feeding at supermaintenance or maintenance levels the average hourly carbon dioxide production during 8-hour periods, and the calculated heat elimination, unaffected by activity, were markedly higher for the low-salt animals than for the controls, although the body weights of the former were, in the majority of cases, less. This effect appeared at the end of the first month and persisted during the last 2 months.

On the basis of comparisons of the heat production including effects of activity the influence of the mineral deficiency appeared to be less marked, but in the same direction.

The increases in heat production due to activity were considerably less for the low-salt animals than they were for their controls.

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OXIDATIVE CHANGES INDUCED BY A MINERAL DEFICIENT DIET [1]

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The proportions of nutrients oxidized in the animal body depend on a number of conditions, prominent among which are the plane of nutrition and the relative proportions of available nutrients in the diet. In the metabolism experiments previously described by Kriss and Smith ('37, '38), involving the use of a diet deficient in minerals, each pair of rats received identical quantities and proportions of organic nutrients, the control ration differing from the low-salt ration only by 4% of mineral salts. In the course of the experiments certain changes in the respiratory quotients were observed in the different feeding periods, indicative of some oxidative changes. It is of scientific interest to know if such changes are related to the mineral deficiency. The present paper deals with this phase of the investigation. The data recorded herein are based on the metabolism experiments cited above.

[1] The data reported in this paper were taken from a dissertation presented by Max Kriss in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1936.

A preliminary report was presented before the American Institute of Nutrition, third meeting, Washington, March, 1936.

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RESULTS

The respiratory quotients

In table 1 are presented the non-protein respiratory quotients of rats receiving the low-salt diet and the control diet, respectively, in the different feeding periods, as outlined in the preceding study (Kriss and Smith, '38). In the computation of these quotients the carbon dioxide production

TABLE 1

Influence of the diets on the non-protein respiratory quotients

RAT NO.	DIET NO.	PERIOD 2	PERIOD 3	PERIOD 4
		R.Q.	*R.Q.*	*R.Q.*
1	I	0.90		
2	II	0.91		
3	I	0.91	0.86	0.90
4	II	0.85	0.92	0.93
6	I	0.89	0.85	0.90
7	II	0.89	0.92	0.95
9	I	0.89	0.90	0.79
10	II	0.89	0.92	0.97
11	I	0.89	0.88	0.90
12	II	0.87	0.93	0.93
13	I	0.87	0.88	
14	II	0.91	0.93	
19	I	0.90	0.92	0.88
20	II	0.87	0.92	0.97
23	I			0.87
24	II			0.89
25	I			0.92
26	II			0.92
Ave.	I	0.89	0.88	0.88
Ave.	II	0.88	0.92	0.94

and the oxygen utilization in the metabolism of protein were calculated from the urinary nitrogen excretion using the factors determined by Kriss and Miller ('34) for the metabolism of casein. These are: 1 gm. of urinary nitrogen equals 5.47 liters of carbon dioxide and 6.67 liters of oxygen.

In period 2, which was approximately a month after the rats were placed on the experiments, no significant differences in the respiratory quotients between the low-salt animals and the controls are noted. The average non-protein R.Q. for the low-salt animals is 0.89 and for the controls it is 0.88. In periods 3 and 4, however, the R.Q. with the low-salt animals is, with one exception in each period, lower than with the controls. The average R.Q. for the rats receiving the low-salt diet is 0.88 in both periods 3 and 4 as compared with 0.92 and 0.94 for the controls.

The differences in the respiratory quotients caused by the mineral deficiency is indicative of effects on the proportions of nutrients oxidized. Since the diets compared showed similar differences between the total and the non-protein R.Q.'s it would seem that the proportions of protein metabolism were not appreciably affected by the mineral deficiency. In order to determine to what extent the lack of minerals influenced the oxidation of the different nutrients table 2 was prepared, which gives the percentage contributions of fat, carbohydrate and protein to the heat production on the two different diets in the various experimental periods.

The partition of the heat production

The data of table 2, which shows the partition of the heat production, are based on the respiratory measurements including effects of activity, the determination of urinary nitrogen and the non-protein R.Q.'s. The heat produced by the metabolism of protein was calculated by the use of the factor 30.59 calories per gram of urinary nitrogen as determined by Kriss and Miller ('34) for the metabolism of casein.

The protein metabolism. The percentage contribution of protein to the heat production in period 2 (table 2), with one exception (rat 6), was somewhat greater with the low-salt animals than with the controls. The average percentage for the low-salt animals is 14.2 and for the controls it is 11.9. In period 3, however, no effect of the mineral deficiency on the

TABLE 2

Percentagee contributions of fat, carbohydrates, and protein to the heat production, as affected by the mineral deficient diet

RAT NO.	DIET NO.	PERIOD 2			PERIOD 3			PERIOD 4		
		Heat produced from			Heat produced from			Heat produced from		
		Fat	Carbohydrates	Protein	Fat	Carbohydrates	Protein	Fat	Carbohydrates	Protein
		%	%	%	%	%	%	%	%	%
1	I	27.9	58.1	14.0						
2	II	25.6	62.0	12.4						
3	I	25.0	60.6	14.4	39.4	46.4	14.2	26.7	55.4	17.9
4	II	43.5	44.8	11.7	22.0	62.9	15.1	19.2	65.6	15.2
6	I	30.8	55.1	14.1	40.8	41.9	17.3	25.7	53.3	21.0
7	II	30.1	54.0	15.9	20.3	58.2	21.5	12.9	67.5	19.6
9	I	30.1	54.1	15.8	27.9	57.9	14.2	59.1	25.2	15.7
10	II	31.1	55.8	13.1	21.8	62.5	15.7	7.9	73.9	18.2
11	I	29.7	53.2	17.1	32.3	50.2	17.5	27.0	56.1	16.9
12	II	37.1	50.3	12.6	18.9	64.9	16.2	18.8	64.4	16.8
13	I	37.3	50.5	12.2	31.2	48.3	20.5			
14	II	26.3	63.7	10.0	18.6	63.7	17.7			
19	I	28.8	59.7	11.5	21.7	62.0	16.3	33.0	51.2	15.8
20	II	39.3	53.2	7.5	22.1	63.1	14.8	8.5	80.1	11.4
23	I							35.5	48.1	16.4
24	II							30.7	55.0	14.3
25	I							22.2	63.5	14.3
26	II							21.9	62.9	15.2
Ave.	I	29.9	55.9	14.2	32.2	54.1	16.7	32.7	50.4	16.9
Ave.	II	33.3	54.8	11.9	20.6	62.6	16.8	17.1	67.1	15.8

percentage of heat derived from protein is apparent, but the value is somewhat higher than in period 2. Similarly, in period 4 there seems to be no significant difference between the contributions of protein to the heat production by the low-salt rats as compared with the controls. The average percentage for the low-salt animals is 16.9 and is only slightly higher than the value (15.8) representing the proportionate protein metabolism of the controls. It is of interest to note that these proportions of protein metabolism with the rats are of about the same order of magnitude as obtain in man (Voit, 1889; Lusk, '28, p. 459), and in cattle (Forbes and Kriss, '32) in normal nutrition.

In general, it may be concluded that the deficiency of mineral salts in the diet did not affect significantly the proportions of protein metabolized although the absolute amounts were apparently affected. This finding is consistent with the fact that the differences between the total and the non-protein respiratory quotients were about alike with the two groups of animals compared.

It appears that the protein metabolism was increased as a result of the mineral deficiency in about the same proportion as was the total heat production. Since the calories derived from protein constitute a relatively small fraction (about 16%) of the total metabolism, therefore the increase in protein metabolism cannot be considered as an important cause for the previously observed increases in the total metabolism and in the basal metabolism. Conversely, it seems likely that the increase in the absolute amounts of protein metabolized was incidental to the increases in intensity of the total metabolism brought about by the deficiency of mineral salts.

The metabolism of fat and carbohydrate. The partition of the non-protein energy between fat and carbohydrates reveals interesting facts regarding the effects of the mineral deficiency on the proportions of these nutrients which were oxidized for energy production. It seems clear from the data in table 2 that after 1 month of feeding the low-salt diet (in

period 2) the proportions of fat and carbohydrates oxidized were not much affected, the differences between the two groups of animals being relatively small. However, in the later periods a marked difference in the proportion of fat oxidized by low-salt animals, as compared with controls, became apparent. Thus, while in period 2 the oxidation of fat by the control animals constitutes, on the average, 33.3% of the total heat production, the average percentage dropped to 20.6 in period 3 and 17.1 in period 4. With the low-salt rats, on the contrary, the average percentage of fat oxidized rose from 29.9%, in period 2, to 32.2% in period 3, and remained practically the same in period 4. The rations in the last two periods were identical.

That the differences indicated above for periods 3 and 4 between the two groups of animals are of unquestionable significance is evidenced by the fact that in both periods 3 and 4 only one pair of animals failed to show a difference between the proportions of fat oxidized. In all other cases the differences are in favor of the low-salt animals. As already stated, the average difference in period 2 is insignificantly small. In this connection it may be mentioned that the low-salt animals are generally characterized by an extreme lack of body fat (Light, Smith, Smith and Anderson, '34).

The effect of the mineral deficiency on the oxidation of carbohydrates is shown to be opposite to the effect on the oxidation of fat. As compared with the controls, the low-salt rats oxidized smaller proportions of carbohydrates in periods 3 and 4.

In general, the proportions of nutrients oxidized by the animals used as controls were less uniform, in the three experimental periods, than was the case with the mineral-deficient animals. Whether the differences noted above in the partition of heat production are due to intrinsic effects of the diet on the oxidative mechanisms in the body, to differences in the voluntary activity of the subjects or to some other factors is a question which needs further investigation. Directly or indirectly these effects seem to be related to the

difference in the mineral content of the diets. The changes in the blood induced by the mineral deficient diet (Swanson and Smith, '32) are probably an important factor in this relation.

The specific dynamic effects of the diets

The conditions of the investigation were not particularly favorable for the determination of the specific dynamic effects of the experimental diets in all the feeding periods. During the course of the investigation marked changes in the basal metabolism of the subjects occurred with advance in age and increase in body weight, while there were no large changes in food intake from one period to another. To compute the specific dynamic effects of the diets in periods 2 and 3 would therefore involve relatively large 'biological errors,' especially since long periods intervened between the determinations of the total metabolism and the measurements of the basal metabolism. For this reason no attempt has been made to determine the specific dynamic effects of the food in these periods.

The determinations of the total metabolism in period 4 (Kriss and Smith, '38) and the final determinations of the basal metabolism (Kriss and Smith, '37) were, however, only 1 day apart. These data, therefore, afforded a basis for the computation of the specific dynamic effects of the diets by direct comparison of the heat production measured in period 4 with the fasting heat production as a base value. The results of this computation are presented in table 3. The use of the fasting heat production as a base value in the computation of the specific dynamic effects of nutrients has been critically discussed by Kriss, Forbes and Miller ('34) and more recently by Kriss ('38). The data of table 3 should be, therefore, viewed in the light of this discussion.

The heat production of the rats in both the feeding and fasting periods (excluding rats 9 and 10, for reasons explained previously) was calculated per 24 hours on the basis of the average hourly heat production unaffected by activity. The increase in heat production in the feeding period

(period 4) over that obtained in the final fasting period is referred to as the specific dynamic effect of the ration. The total heat increments, expressed in kilogram-calories, are shown by the column of figures next to the last. In the last column the heat increments (specific dynamic effects) are expressed as calories per gram of digestible organic matter; it is seen that they exhibit very large variations.

TABLE 3

The specific dynamic effects of the diets

PERIOD 4						
RAT NO.	Diet		Metabolism per 24 hours		Heat increment	
	No.	Digestible organic matter	Total	Basal	Total	Per gram digestible organic matter
		gm.	*cal.*[1]	*cal.*[1]	*cal.*[1]	*cal.*[1]
3	I	4.1	181.9	182.4		
4	II	4.0	178.3	158.2	20.1	5.0
6	I	4.6	184.3	178.3	6.0	1.3
7	II	4.6	168.4	163.9	4.5	1.0
11	I	5.0	212.6	179.5	33.1	6.6
12	II	4.9	212.4	167.0	45.4	9.3
19	I	4.1	199.0	177.6	21.4	5.2
23	I	4.1	190.6	173.5	17.1	4.2
24	II	4.0	169.7	149.3	20.4	5.1
25	I	4.0	191.0	168.0	23.0	5.7
26	II	4.0	166.8	151.4	15.4	3.9

[1] Kilogram-calories.

In comparing the dynamic effects of the low-salt diet with those of the control diet it is observed that in three of the five comparisons the low-salt diet shows the smaller effect, while in the other two the opposite is true. These differences cannot, therefore, be considered as significant. It appears that the increases in the basal metabolism caused by the mineral deficiency can fully account for the relatively greater increases in the total heat production of the low-salt rats in the feeding periods.

SUMMARY

The respiratory quotients of rats receiving a diet deficient in inorganic salts were almost identical with those of control animals during the first month on the diets. During the second and third months, however, the respiratory quotients of the low-salt animals were significantly lower than those of their controls. The mineral deficiency apparently resulted in the oxidation of relatively larger proportions of fat and smaller proportions of carbohydrates, while it was practically without effect on the protein metabolized.

In spite of the differences in the total heat production there were no significant differences between the specific dynamic effects of the diets compared, as these effects are generally defined. This is explained by the fact that the increases in the total metabolism caused by the mineral deficiency can be fully accounted for by similar increases in the basal metabolism.

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ANOMALIES IN THE BIO-ASSAY OF VITAMIN D MILK

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The standard method for the bio-assay of vitamin D depends on susceptibility of the albino rat to the rachitogenic processes under a strictly limited dietary regime and the subsequent reversal of the processes on administration of graded amounts of the vitamin. The use of the albino rat for this purpose is, in certain respects, unfortunate. In this species, the addition of vitamin D or its withdrawal from an otherwise normal diet appears to exert only a minimum amount of influence on the conditions which constitute the criteria of the test. Of primary importance to the rat, apparently, are not only the Ca : P ratio in the diet, but also the absolute levels of these elements in the food. Furthermore, according to Shohl ('37), the total acid-base balance of the diet is a very critical factor in determining the rachitogenic power. It has also been shown by Shohl that certain organic acids and their alkali salts (notably citrates) may exert a striking effect on the production and healing of rickets quite independently from the calcium, phosphorus or vitamin D content of the diet.

The technic commonly employed for the estimation of vitamin D was developed in connection with the testing of fish-liver oils and their non-saponifiable fractions, since these materials were the first potent sources of the vitamin to be recognized. Such supplements added to the diet amount to only a very small portion of the total caloric intake and, in

addition, carry almost no mineral contaminants. Consequently, in their assay, the constancy of the experimental diet may be maintained with reasonable accuracy. The application of the method to more complex natural food materials must necessarily entail considerable juggling of the diet mixtures if a serious attempt is made to maintain a constant intake of all elements other than the vitamin itself.

The assay of vitamin D milks has not yet received sufficient critical study. It is understood that the Committee on Standard Methods for the United States Pharmacopoeia has an investigation of the problem under way but, as yet, only preliminary reports have appeared (Russell, '37). In the preliminary report of the committee's chairman (H. T. Scott, November, 1937), the procedure outlined is described as purely advisory and no mention is made of any possible effects of milk constituents on the apparent potency of the test materials. We have been informed (personal communication) that this point is receiving attention in the laboratory of one of the members of the Committee on Standard Methods (W. C. Russell) and that the findings will be published shortly.

The species specificity of the several known vitamins D has now been well established (Bills, '37). Since, depending on the method of preparation, the antirachitic activity of milk may be of either animal or vegetable origin and, since vitamins D from these two sources appear to constitute groups of distinct physiological activity, a certain amount of confusion may arise in assay work due to the differences between these two types of antirachitic agents. The status of vitamin D milks in the nutrition of the human infant has been critically reviewed by Jeans ('36).

It has been contended, almost from the beginning of vitamin D research, that milk is peculiarly suited to the role of a vitamin D carrier. It undoubtedly offers certain practical advantages to the pediatrician as a convenient means of prescribing vitamin D. Furthermore, the administration of the vitamin in close association with easily assimilable calcium and phosphorus has been advanced by some as an advantage

on theoretical grounds. Reports of experiments designed to test such a theory are not numerous. Obviously, comparable data can be obtained only by a series of tests on the same species with the same form of vitamin D, the only variable factor being the medium in which the vitamin is administered. Results obtained with one species of test animal are too difficult to evaluate in terms of results given by another species.

Lewis ('35), in a series of studies on rachitic children, reported that 150 I.U. of crystalline vitamin D in milk appeared to give as favorable responses as 300 I.U. in oil. Later, he reported (Lewis, '36) prophylactic studies in which infants receiving crystalline vitamin D in milk developed rickets less frequently than those receiving the same number of units in corn oil. Shelling ('37 a, b) has also reported curative studies on infants from which he concluded that viosterol emulsified in milk was about ten times as effective, unit for unit, as when given in oil. Jeans and Stearns ('34), however, could find no significant differences between the calcium retentions of infants receiving cod liver oil and cod liver oil concentrate (Zucker) in milk on a unit for unit basis.

Incidental to a study of the response of the chick to various forms of vitamin D, Haman and Steenbock ('35) could find no evidence that milk constituents, other than vitamin D, had any influence on the antirachitic effectiveness for this species. For the rat, on the other hand, Supplee, Ansbacher, Bender and Flanigan ('36) were able to show a very marked enhancement of the potency of crystalline vitamin D when incorporated in skimmed milk or whole milk. On the basis of their experiments, they attributed this enhancement to the formation of a 'symplex' between the vitamin and the lactalbumin of the milk, the symplex being, presumably, more readily utilized by the animal.

EXPERIMENTAL

Throughout this work, the source of the skimmed milk was whole, raw Guernsey milk from the same herd. The milk was separated within an hour of milking in a Sharples supercen-

trifuge at 36°C. The bowl was fitted with a no. 8¾ ring dam and was run at 35,000 to 40,000 r.p.m. Butter-fat determinations (Babcock and Roese-Gottlieb) on the skimmed milk gave values between 0.01 and 0.03%.

The bio-assays were done by the 10-day curative method (8-day experimental feeding period) using the radiographic technic for estimating healing (O'Brien and Morgareidge, '34, '38). Wistar strain albino rats were used, each test group consisting of seven to ten animals. The materials to be tested were always fed to several groups of rats at different levels and the antirachitic effect compared with that shown by the international standard solution of vitamin D in several groups of rats treated simultaneously. Since usually about 100 rats were used at one time, the same series of standard animals were made to serve for several experiments. The rachitogenic diet used throughout was the no. 2965 ration of Steenbock and Black ('25).

The skimmed milk was tested for its effect on the apparent antirachitic potency of two different concentrates in addition to the international standard solution of irradiated ergosterol. The two concentrates, Vitex [1] and UVO,[2] are commercial preparations available to the dairy industry for the production of vitamin D milks. Vitex consists of a cod liver oil concentrate (Zucker process) homogenized in cream. The sample designated VD had a nominal potency of 750 I.U. per gram. Samples VA_1 and VA_2 were rated at 3000 I.U. per gram. UVO is a similar concentrate prepared from irradiated ergosterol and has a nominal potency of 4000 I.U. per gram.

Both of these materials were assayed in three different ways. First, the concentrate was fed in the minimum amount (100 mg.) of skimmed milk which could be conveniently administered from calibrated dropping pipettes. Second, the concentrate was fed in the same manner and dosage as in the first type of experiment but, in addition, 4.0 cc. of skimmed milk were fed daily in individual dishes during the test feeding period. Third, the concentrate was fed as fortified skimmed milk which was so prepared that the daily ration of 4.0 cc. contained the proper dosage dispersed in it.

[1] The sample of Vitex-VD was furnished through the courtesy of Dr. Andre E. Briod of the National Oil Products Company, Vitamin Division, Harrison, New Jersey. Samples VA_1 and VA_2 were purchased from a local dairy which retails Vitex fortified vitamin D milk.

[2] The UVO concentrate was prepared by the Wisconsin Alumni Research Foundation, Madison, Wisconsin. We are indebted to Dr. H. T. Scott for supplying this sample.

The results of a series of experiments including these three types of test feeding are summarized in table 1. It will be seen that the values found in experiments 1 to 6 correspond quite closely with the nominal potencies as stated by the manufacturers (average, 111%). In experiments 8 to 11, however, where 4.0 cc. of untreated skimmed milk were fed simultaneously with, but separate from the concentrate, an average of 185% of the nominal values was found. Experiment 7 shows this effect for the international standard solution itself. The results of experiments 12 to 18 indicate that an even greater effect is obtained from the fortified skimmed milk. Here, the average value found is 293% of the nominal potency, although experiment 12 is excluded from the average since it appears to be anomalous. At the present time, no very convincing reason can be offered as to why this dispersion or dilution effect occurs. It does appear quite real, however, and further work is now in progress on this point.

The effects of adding small amounts of phosphorus (as sodium glycerophosphate) to the fortified skimmed milk are shown in experiments 19 to 21. This milk was the same sample which assayed 200% of nominal potency in experiment 18. Thus 4 mg. of phosphorus in the daily allotment of milk resulted in increasing this apparent potency to 400% above the nominal value.

Consideration of these results leads one to wonder what part of the over-all antirachitic potency of vitamin D milks may be extractable by means of usual lipid extraction methods since the known forms of this vitamin are soluble in the fat solvents. Therefore, lipid extracts have been prepared from both irradiated and fortified skimmed milks because it seemed reasonable to suppose that they would contain all the actual vitamin D present and that a determination of the potency of these extracts would give some measure of any possible enhancing action of the other milk constituents.

To this end, the following procedure was adopted: The milk was run slowly from a separatory funnel (through a stopper fitted with a high-speed stirrer) into a balloon flask containing six volumes of boiling 95% alcohol. After the addition was complete, the boiling was continued for 10 minutes and the protein-lactose precipitate filtered off immediately on fluted no. 50 Whatman papers. The precipitate was resuspended in a convenient volume of boiling alcohol and again filtered. The combined filtrates were diluted with distilled water until the alcohol concentration was 50% and then extracted in a large

TABLE 1

The effect of skimmed milk on the apparent potency of various antirachitics

EXPERIMENT NO.	ASSAY NO.	ANTIRACHITIC SUBSTANCE	NOMINAL POTENCY	HOW FED [1]	TOTAL ANIMALS COMPARED	ASSAY LEVEL INT. STD.	EQUIVALENT LEVEL TEST SUBSTANCE	APPARENT POTENCY	'ENHANCEMENT' PER CENT OF NOMINAL
			I.U./gm.			*I.U.*	*mg.*	*I.U./gm.*	
1	57	Vitex-VD	750	—Sk	45	0.35	0.57	615	82
2	58	Vitex-VD	750	—Sk	42	0.70	0.64	1090	145
3	60	Vitex-VD	750	—Sk	49	0.40	0.50	800	107
4	63	Vitex-VA_3	3000	—Sk	42	0.57	0.18	3170	106
5	61	UVO	4000	—Sk	49	0.40	0.10	4000	100
6	62	UVO	4000	—Sk	42	0.57	0.114	5000	125
								Average	111
7	49	Int. Std.	1000	+Sk	54	1.00	0.625	1600	160
8	58	Vitex-VD	750	+Sk	49	0.70	0.33	2100	280
9	60	Vitex-VD	750	+Sk	42	0.40	0.31	1300	170
10	63	Vitex-VA_3	3000	+Sk	42	0.81	0.15	5400	180
11	62	UVO	4000	+Sk	42	0.57	0.105	5400	135
								Average	185
12	57	Vitex-VD	750	FSk	45	0.70	0.07	10,000	1330
13	58	Vitex-VD	750	FSk	49	0.35	0.105	3300	430
14	60	Vitex-VD	750	FSk	49	0.40	0.20	2000	266
15	55	Vitex-VA_3	3000	FSk	64	0.70	0.09	7800	260
16	63	Vitex-VA_3	3000	FSk	57	0.57	0.06	9500	316
17	61	UVO	4000	FSk	49	0.57	0.05	11,400	285
18	62	UVO	4000	FSk	42	0.40	0.05	8000	200
								Average	293
19	62	UVO + P (1 mg.)	4000	FSk	21	0.57	0.057	10,000	250
20	62	UVO + P (2 mg.)	4000	FSk	21	0.57	0.038	15,000	375
21	62	UVO + P (4 mg.)	4000	FSk	21	0.57	0.036	16,000	400

[1] —Sk denotes that the supplement was fed in 100 mg. of skimmed milk daily.

+Sk denotes that a daily ration of 4.0 cc. of skimmed milk was fed in addition to, and separate from the supplement.

FSk denotes that the daily ration of 4.0 cc. of skimmed milk was fortified directly by the dispersion of the supplement in it. The average per cent of nominal potency found for this group does not include experiment 12, the results of which appear to be anomalous.

Experiments 19, 20 and 21 are assays of the same sample, run at the same time, as that shown in experiment 18 and show the effect of added phosphorus dispersed in the milk in addition to the antirachitic supplement. Sodium glycerophosphate was added to the milk in amounts calculated to give the phosphorus increments indicated in the table.

The total number of animals compared was 916. However, the same set of standard animals served all those experiments having the same assay number.

continuous extractor with redistilled petroleum ether (b.p. 35 to 40°C.) for 24 to 36 hours. The petroleum ether was evaporated off in vacuum and the oily residue taken up in corn oil for feeding via calibrated dropping pipettes to rats. An alternate method consisted in reducing the alcohol filtrate to a convenient volume for extracting by hand in separatory funnels. For this purpose, a vacuum still was used operating at 35°C. with a small nitrogen bleeder into the vapor space. Traces of oxygen were removed from the nitrogen by passage through hot copper gauze packed tightly into a glazed quartz tube. The protein-lactose precipitate gave consistently negative results when assayed for vitamin D activity.

By either of these methods, an average of 650 mg. of lipid material could be extracted from 1 liter of skimmed milk. From this it is evident that both the Roese-Gottlieb and Babcock methods indicate about one-third of the total lipid extractable with hot alcohol.

The antirachitic potency recoverable in these lipid extracts from both irradiated and fortified skimmed milk is in line, in order of magnitude, with the enhancement effect. Quantitative results, however, are not yet ready for publication and will be reported later.

DISCUSSION

It seems reasonably apparent that the first conclusion to be drawn from these data is that the bio-assay of vitamin D may not always be a measure of the actual vitamin (considered as a chemical entity) but only of an over-all response of the experimental animal to a set of nutritional factors. The results show definitely that feeding skimmed milk in addition to the vitamin containing substance results in an increase of the antirachitic effectiveness and that feeding the same material intimately dispersed in the same volume of milk results in a much greater effect. Thus it appears that not only does the mere presence of milk in the diet facilitate the action of vitamin D, but also that this enhancement is dependent to a marked degree on the physical relations between the two factors in the gastro-intestinal tract. As measured by subsequent bone formation, at least, the absorption of calcium or phosphorus, or both (depending on which happens to be the limit-

ing factor), is greatly favored when presented to the gut wall in close association with vitamin D.

The role of vitamin D in calcium and phosphorus absorption in the rat has been recently studied by Nicolaysen ('36, '37 a). He concluded that vitamin D is little, if at all, concerned in the absorption of phosphorus but that, in the gut, the effect of vitamin D is confined to a direct action on the absorption of calcium. The well-known reduced absorption of phosphorus in vitamin D deficiency is due, according to Nicolaysen, to a precipitation by the increased amount of calcium in the intestine.

Just why milk calcium should be more readily absorbable in the presence of vitamin D than the calcium already present in the diet in large amount is not easily explained. It should be borne in mind that it is by no means proved that vitamin D action is limited entirely to the gut. In fact, that such is not the case is indicated by the finding (Nicolaysen, '37 b) that rachitic rats on the no. 2965 diet absorb twice their actual daily requirement of calcium and only about 30% of their phosphorus needs. Furthemore, Schneider and Steenbock ('38) have shown that in rats made rachitic on a special diet very low in phosphorus, vitamin D induces healing of the rickets but completely inhibits body growth. They feel that under the stimulus of the vitamin, all available phosphorus becomes 'ear-marked' for bone formation leaving insufficient amounts for the new formation of soft tissues. Taken together, these two observations would seem to indicate a specific action for vitamin D in the bone forming system in addition to whatever role it may play in intestinal absorption.

Young rats on the no. 2965 diet consume about 7 gm. of food per day for an average weight gain of 1 gm. According to the calculations of Nicolaysen, for each 1 gm. increment in body weight, 4.5 mg. of phosphorus must be absorbed and utilized, and he has shown that vitamin D deficient rats are able to absorb only about 30% of this amount from the diet. But Lowe and Steenbock ('36) have found that of the 300 mg.

of total phosphorus per 100 gm. of this diet, only about 6% is inorganic, the remainder being, mainly, poorly absorbable inositol hexaphosphate. Consequently, the daily intake of available phosphorus on this diet does not amount to more than about 1.3 mg. The values to be found for the phosphorus content of milk range between 85 and 197 mg. per 100 gm. Thus, as a rough approximation, we may assume that 4 cc. of skimmed milk will contain between 4 and 5 mg. of phosphorus, most of which has been generally assumed to be readily absorbable.

Evidence that this additional phosphorus intake which occurs in the assay of milk samples can have a marked influence on the apparent potency of the samples is seen in the results of our experiments 19 to 21. It would seem quite possible that a considerable part of the variation in the enhancement effect seen from assay to assay may have been due to normal variations in the phosphorus content of the milk used.

One other factor should be mentioned in this discussion. The role played by lactose in the nutrition of the growing animal may well have some special significance. A number of studies has been reported in which lactose ingestion by normal and rachitic animals has been related to the degree of calcification or healing. Kline, Keenan, Elvehjem and Hart ('32) found that adding 40% of lactose to the diet of rachitic chicks resulted in nearly normal bone development. Addition of vitamin D by the direct ultraviolet irradiation of the chicks produced a higher ash content of the bones than did lactose alone. They concluded that lactose acts as a supplement to vitamin D in calcium assimilation, possibly through its effect on the pH of the gut. Outhouse, Smith, Merritt and White ('37) have found that a daily lactose intake by growing rats equivalent to that contained in 30 cc. of cow's milk (about 1.2 gm.) results in bone ash values which approach but do not equal those attainable with cod liver oil. Since our own tests have shown that as much as 10 cc. of skimmed milk daily for 8 consecutive days (without vitamin D) have no effect on

the healing of rickets in rats, we felt, at the time these experiments were planned, that the amount of lactose in 4 cc. of milk would not be an important factor. The possibility that this sugar may exert a synergistic effect with vitamin D, even when fed in amounts too small to be effective alone, is being taken into account in the planning of future experiments.

SUMMARY

Experiments have been described which show that skimmed milk which has been fortified with vitamin D by the addition of concentrates exhibits, on bio-assay, an enhanced apparent potency which is approximately three times greater than the value expected, based on the amount of concentrate added. Furthermore, it is shown that feeding the concentrate and the skimmed milk separately to the same rats results in less enhancement of potency than does feeding the concentrate in the form of fortified skimmed milk. Milk also enhances the potency of the international standard solution of vitamin D.

In discussing these results, it is pointed out that the response of the rachitic albino rat to certain dietary factors other than vitamin D is well known. The bio-assay of vitamin D milk may introduce several of these factors. In view of the conditions imposed by the usual curative assay procedure, the roles played by phosphorus and, possibly, lactose in the effective antirachitic action of vitamin D milk appear to deserve special attention in the future work on this problem.

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